CHAPTER 1

INTRODUCTION

1.1. DIABETES MELLITUS

1.1.1. Definition and Overview

Diabetes mellitus (DM) is a condition whereby the metabolism and utilisation of glucose is impaired, leading to elevated glucose levels in the plasma, termed hyperglycaemia. The terms are Greek in origin; *diabetes* means "to pass through" and *mellitus* means "sweet urine", which summarises the observation that patients usually pass large quantities of sweet urine, due to the presence of glucose in it.

DM is defined by the World Health Organisation (WHO) as a chronic disease that occurs when the pancreas does not produce enough insulin, or when the body cannot effectively use the insulin it produces. Insulin is the enzyme that facilitates glucose uptake from the plasma into cells via insulin receptors that are present on all cells. Deficiency or absence of insulin leads to impaired utilisation of glucose, and subsequently to hyperglycaemia.

A hyperglycaemic state is unfavoured by the body. As such, the acute effect of hyperglycaemia is the result of the body trying to correct this condition. For one, there is osmotic diuresis in which the kidneys try to excrete as much glucose as possible from the body; this leads to glycosuria (H. W. E. Chan, Ashan, Jayasekera, Collier, & Ghosh, 2012). Furthermore, as tissues are deprived of glucose, their main source of energy, a catabolic process occurs in which the body tries to compensate for this apparent deficiency by breaking down amino acids and fats, and increasing insulin secretion thus stimulating appetite to increase intake of glucose (Giaccari, Sorice, & Muscogiuri, 2009). All of these processes are reflected in the classical clinical picture of DM: polyuria (increased urination due to the diuresis), polydipsia (excessive water

consumption to counter the water loss) and polyphagia (increased food intake due to the body's perception of lack of glucose), in association with lethargy and weight loss.

Prolonged hyperglycaemia causes macrovascular and microvascular complications due to direct glucotoxicity (Cao, Chen, Wang, Liu, & Liu, 2011; Correia et al., 2012; Sugiyama et al., 2011), inflammation (Bryland, Wieslander, Carlsson, Hellmark, & Godaly, 2012; T. N. Kim et al., 2010; Noratto, Angel-Morales, Talcott, & Mertens-Talcott, 2011) and increased oxidative stress (Bryland, et al., 2012; Srinivasan & Pari, 2012). Macrovascular problems that may arise stem from accelerated atherosclerosis due to endothelial cell dysfunction, leading to hypertension, ischaemic heart disease (Mazzone, Chait, & Plutzky, 2008) and cerebrovascular disease (Mulnier et al., 2006). Damage to the microvasculature is more extensive, with involvement of the ophthalmic, renal (Weijers & van Merode, 2001) and peripheral nervous systems (Voulgari et al., 2010). All these translate into a reduced quality (Chyun et al., 2006) and expectancy of life for diabetes sufferers.

1.1.2. Classification of Diabetes Mellitus

There are two major types of diabetes mellitus, both with distinct pathophysiologies, affecting different age groups and presenting initially with different spectrum of symptoms. Type 1 diabetes mellitus (T1DM) is an autoimmune disease, in which autoantibodies against the pancreatic beta cells effectively destroy them. Formerly known as insulin-dependent diabetes mellitus, it occurs early in life due to the failure of pancreatic beta-cells to produce an adequate amount of insulin. At the onset, patients usually present in a state of acute severe insulin deficiency called diabetic ketoacidosis (DKA). Insulin replacement therapy is the mainstay of treatment in this group of patients (Gan, Albanese-O'Neill, & Haller, 2012). T1DM constitutes around ten percent of overall DM cases.

The most common type of DM is type 2 DM (T2DM), which comprises around 85% of overall DM cases. In this type, there is a relative insulin deficiency leading to chronic, usually post-prandial, hyperglycaemia. The pathogenesis of T2DM begins with reduced insulin sensitivity by cells and eventually leading to pancreatic beta-cell dysfunction and failure (Nolan, Damm, & Prentki, 2011). T2DM is mainly a lifestyle disease, where excess consumption of glucose in the diet is not balanced by adequate utilisation by cells, due to physical inactivity. The onset of the disease is more transient, where patients would normally present with non-specific symptoms like lethargy and weight loss despite eating more, and/or specific ones like polyuria and polydipsia. Treatment is both pharmacological-based and lifestyle modification; the former targeted directly at reducing hyperglycaemia, the latter to improve insulin sensitivity and utilisation.

The remaining five percent of cases are due to other types of diabetes such as gestational diabetes, drug-induced secondary diabetes (of which oral corticosteroids are the main culprit) and monogenic types of diabetes affecting either beta-cell function or insulin action on the tissues. For the purpose of discussion of this thesis, the sole focus is on T2DM.

1.1.3. Pathogenesis of T2DM

The aetiology behind T2DM is multifactorial and sometimes dissimilar between patients. However, the final common pathway remains the same: elevation of plasma glucose concentration due to defective insulin production and action. Chronologically, defective insulin action and compensatory hyperinsulinaemia are early features of the disease. The latter is an attempt by the pancreas to maintain normoglycaemia. Nevertheless this process is a vicious cycle and eventually, the pancreas will be

exhausted in its attempt to satisfy the demands of the body for an increasing amount of insulin; beta-cell dysfunction and failure ensues.

Why does the body react to hyperglycaemia by reducing insulin action? Insulin acts by promoting glucose uptake into cells. Upon binding onto its cell surface receptor, insulin triggers the autophosphorylation of the receptor, leading to the activation of its tyrosine kinase enzyme. This enzyme in turn mediates the tyrosine phosphorylation of insulin receptor substrates (IRSs, e.g. IRS1-4, Gab1 and Shc). Via downstream interactions, the IRSs promote translocation of a glucose receptor called GLUT-4 from its intracellular vesicles to the plasma membrane of the cells. GLUT-4 promotes glucose uptake by the cells.

Excessive intracellular uptake of glucose is detrimental to the cells, by way of increased apoptosis of glucose-laden cells (Kluth et al., 2011). There exist built-in defence mechanisms that prevent this eventuality. Hyperglycaemia causes a number of physiological and pathological processes to take place: activation of the hexosamine pathway, increased oxidative stress, elevation of intracellular protein kinase-C (PKC) levels, induction of inflammation and several other cellular and biochemical events (Giaccari, et al., 2009). The above processes reduce insulin sensitivity by impairing tyrosine phosphorylation of the IRSs (specifically, IRS-1), leading to a reduction in phosphoinositide 3-kinase (PI3K) activity which is a key component in facilitating GLUT-4 translocation to the plasma membrane of cells.

Overt T2DM at this stage is usually averted by compensatory hyperinsulinaemia, putting considerable strain on the productive and excretive capacity of the pancreatic beta-cells. At this juncture, symptoms of T2DM are not yet evident in the patients. However, elevated plasma glucose levels are deleterious to the beta-cells, as demonstrated in an animal model (Kluth, et al., 2011). In time, the beta-cells succumb to these stimuli, undergo accelerated apoptosis and experience a reduction in

mass (Lupi & Del Prato, 2008). Beta-cell dysfunction ensues, followed closely by symptoms of decompensation (i.e. polyuria, polydipsia and polyphagia) and a diagnosis of T2DM.

There are two types of hyperglycaemia in T2DM: fasting hyperglycaemia and postprandial hyperglycaemia. Fasting hyperglycaemia in T2DM patients is due to increased glycogenolysis and gluconeogenesis by the liver, in response to a defective suppression of glucagon (Rizza, 2010). Postprandial hyperglycaemia is more damaging. In normal individuals, increasing glucose concentration after meals is rapidly followed by release of insulin (acute phase insulin release) causing glucose concentration to fall to pre-prandial levels within two hours of the meal. This acute phase insulin release is non-existent in T2DM, leading to a delayed insulin response and postprandial hyperglycaemia (Butler & Rizza, 1991). Furthermore, it was noted that only oral glucose intake stimulates the acute phase insulin release. This pattern is not observed with parenterally administered glucose, leading to the discovery of the entero-insular pathway which is mediated by the incretin hormones (Drucker & Nauck, 2006).

1.1.4. Incretins in T2DM Pathogenesis

There are two incretin hormones important in modulation of hyperglycaemia: glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). They are part of the enteroinsular or incretin pathway, which promotes the acute phase, glucose-stimulated insulin secretion. GIP and GLP-1 are excreted by intestinal cells into the portal circulation in response to nutrients detected in the intestinal lumen even before absorption has taken place. Stimulation of the pancreatic beta-cells by these hormones leads to an ameliorated secretion of insulin during oral ingestion of glucose (Campioni et al., 2007).

GIP is a 42 amino-acid peptide, of which the gene is located on chromosome 17 in human. It is produced by K cells in the upper small intestines. GIP is secreted by the duodenum and proximal jejunum in response to oral ingestion of carbohydrates and lipids.

GLP-1, as the name suggest, is a product of the pro-glucagon gene, which is located on the long arm of chromosome 2. Other products of this gene are glucagon, GLP-2 and other pro-glucagon derived peptide. GLP-1 is mainly expressed in mucosal L cells in the ileum and colon, and is secreted in response to oral ingestion of carbohydrates. It is also expressed outside of the gastro-intestinal tract, namely in pancreatic alpha cells as well as neurons in the brain regions of hypothalamus, pituitary, nucleus of the tractus solitaris and reticular nucleus. Despite the distal location of the L cells, GLP-1 release is prompt and is thought to be indirectly controlled via neural and endocrine methods, rather than by direct contact of nutrients with the cells.

Both GIP and GLP-1 have very short half-lives of five to seven and one to two minutes respectively, as they are rapidly metabolised by the enzyme dipeptidyl peptidase-IV (DPP-IV) that is present in and on all cells. Upon reaching the pancreatic beta cells, their effects are mediated via specific receptors present on the cellular membrane. These receptors are G-protein coupled, with the final pathway leading to enhanced exocytosis of insulin-containing granules from the beta cells (reviewed by (Gautier, Choukem, & Girard, 2008)). Apart from this immediate insulinotropic action, GLP-1 also enhances insulin synthesis. Moreover, GLP-1 has a cytoprotective effect on the beta-cells themselves, promoting growth and regeneration whilst preventing apoptosis (Drucker & Nauck, 2006).

Besides pancreatic beta cells, GLP-1 receptors are also present on extrapancreatic tissues such as the central and peripheral nervous systems, heart, lungs, stomach, kidneys, adipose tissues and skeletal muscle. The effects of GLP-1 on these

tissues favour an antidiabetic state for the body, i.e. reduced appetite, increased glucose uptake and glycogen synthesis, and delayed gastric emptying. GIP receptor distribution is not as generalised as that of GLP-1, as most of it is found on beta-cells, with a much lesser disposition in adipose tissue and central nervous system. Hence, GIP's glucose-lowering effect is limited to just its insulinotropic property (Drucker & Nauck, 2006).

It is conceivable how dysfunction(s) in the entero-insular axis can give rise to the development of T2DM. It has been proven that in T2DM individuals, the incretin effect is blunted, either due to an impaired secretion of GLP-1 and/or a reduction of GIP action (Gautier, et al., 2008). These impairments lead to postprandial hyperglycaemia. Although in theory both peptides work in tandem to ensure normoglycaemia, due to GLP-1's extra-pancreatic effects, most research are concentrated on evaluating this peptide.

1.1.5. Epidemiology of DM

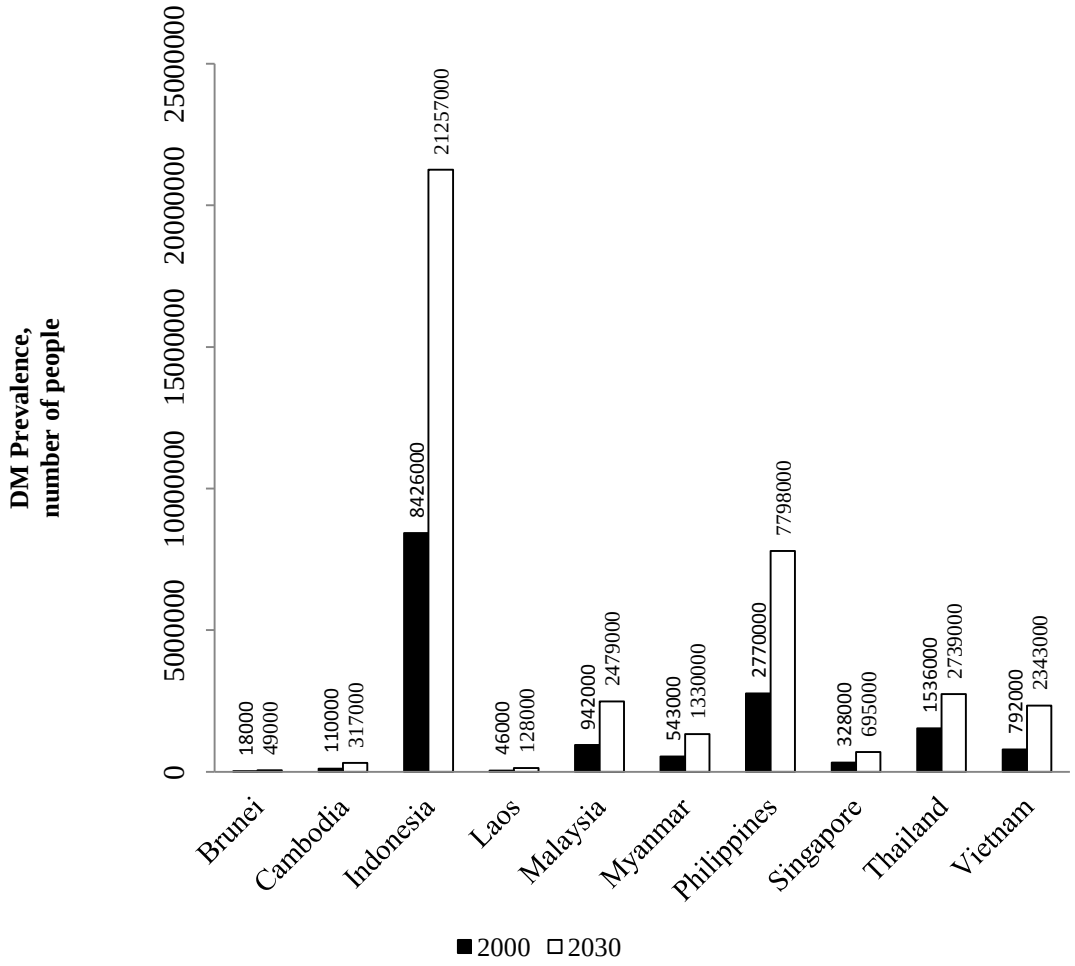
Historically, researchers in the 1970s have predicted that lifestyle diseases, especially T2DM will reach epidemic proportions (Laakso, 2003). Globally, the prevalence of DM in 2000 was estimated by WHO at 171 million people, with the most number of sufferers located in the South East Asian region (46.9 million people). This number is expected to increase to 366 million within the span of 30 years, with the South East Asian region recording the most number of cases (119.5 million) (Wild, Roglic, Green, Sicree, & King, 2004). As T2DM constitutes 75 to 80 percent of overall DM cases, it is expected that there will be around 300 million T2DM sufferers globally in 2030.

According to WHO, countries in the South East Asian region include Bangladesh, Bhutan, Democratic People's Republic of Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka and Thailand. Malaysia is listed to be in the

Western Pacific region. Most of these countries are developing nations, which are currently experiencing a shift from an agricultural-based economy to a more industrialised-based one. This leads to a decrease in physical activity as labour-intensive farming is replaced by machine-operating or office-based jobs. The increased availability of refined food, coupled with an influx of westernised fast-food operators into these regions, lead to overeating and an increase in obesity prevalence. All these factors contribute to the higher development rate of T2DM in this region.

With regards to Malaysia, the national prevalence of T2DM in adults above the age of 30 years in 2006 is 14.9 percent according to the 3rd National Health and Morbidity Survey, NHMS III, which is translated to be around 3 million people. The prevalence of DM among ASEAN countries is summarised in Figure 1.1. As WHO estimated that Malaysia will have 2.5 million DM sufferer in 2030, the prevalence stated in NHMS III has already surpassed that estimation, with 14 years to spare. This is due to an increasing number of people adopting a sedentary lifestyle leading to an increased prevalence of obesity, the major risk factor for developing T2DM (J. C. Chan et al., 2009; Hussain, Claussen, Ramachandran, & Williams, 2007). Obesity prevalence in Malaysia has steadily risen; from 5.5% in 1996 to 14% in 2006 (Khambalia & Seen, 2010), which is reflected in the rest of Asia (Ramachandran & Snehalatha, 2010). Although in a Caucasian population, the rise in obesity only partly explains for the rise in T2DM prevalence (Hardoon et al., 2010), the same cannot be said for an Asian population, as reflected by the concurrent rise in both obesity and T2DM in this region.

Figure 1.1: Prevalence of DM in the years 2000 and 2030 in ASEAN countries



1.1.6. Risk Factors for T2DM Development

T2DM is a multifactorial disease, with complex interactions between environmental and genetic factors resulting in impaired glucose metabolism. Traditional risk factors are obesity, nutritional status, lifestyle changes, smoking and pancreatic beta cell function, which are inter-related. Genetic risk factors are mutations, epigenetic changes and genetic polymorphisms. The most important independent risk factor is obesity. Obesity is defined as having a body mass index (BMI) of more than 25kg/m^2, with severe obesity defined as having a BMI of more than 30kg/m^2. Obesity is associated with an elevated level of glucose, triglycerides and total cholesterol levels (Jensen, 2008), as well as biochemical markers for inflammation (Rasouli & Kern, 2008). The obese state also predisposes an individual to a reduction in insulin sensitivity – a defence mechanism in the state of persistent recurrent hyperglycaemia. As previously detailed, this eventually leads to T2DM development.

Abdominal obesity is particularly important, as it is related to lower muscle mass and an increased disposition of fat around internal organs (i.e. visceral adiposity), both of which will predispose to T2DM. Asians are particularly susceptible to visceral adiposity, with studies showing that at the same BMI, Asians have a higher amount of visceral fat than Europeans (Lear, Humphries, Kohli, & Birmingham, 2007; Lear et al., 2007). Although the emphasis is on the prevention of abdominal or visceral adiposity, generalised obesity also has an impact on the functioning of the pancreatic beta-cells. Small alterations in BMI were shown to impact on the secretory function of the beta-cells disproportionately to the level of BMI change as well as to the level of insulin sensitivity observed (Funakoshi et al., 2008).

The nutritional status of individuals, especially when comparing urban with rural areas, is rapidly being altered due to socioeconomic development, favouring overeating and a sedentary lifestyle. Furthermore, the consumption of high levels of trans-fatty

acids in certain types of food has also been implicated in increasing T2DM risk. In general, increased consumption of food with a high glycaemic index doubles the risk of T2DM. Furthermore, changes in socioeconomic scenario also lead to other alterations in lifestyle. Increasing urbanisation leads to a reduction in sleeping hours, an increase in psychosocial stresses and an increase in the risk of depression. Depression has been proven to be an independent risk factor for T2DM and associated with poorer glycaemic control (Heckbert et al., 2010).

Urbanisation also brings with it another risk factor for T2DM, i.e. an increasing number of smokers. Smoking has been associated with a 44% increase in T2DM risk (Cho et al., 2009), due to induction of insulin resistance and the subsequent impairment of compensatory increase in the insulin secretory response (Chiolero, Faeh, Paccaud, & Cornuz, 2008). Smokers are also more likely to have abdominal obesity, with its risk explained previously.

Environmental risk factors do not completely explain the increasing incidence of T2DM, as there is interaction between the aforementioned factors with another component: genetic susceptibility of individuals (Qi, Cornelis, Zhang, van Dam, & Hu, 2009; Romao & Roth, 2008). The genetic component is by far the most important risk factor simply because it is an unmodifiable risk, together with others such as gender (males more susceptible to T2DM than females), advancing age and ethnicity (South Asians more susceptible than Chinese) (Khan et al., 2011).

There are several genetic abnormalities that might predispose to the development of DM. Specific mutations in certain genes affect functioning of the pancreatic beta-cells, insulin signalling pathway and glucose/insulin receptors have led to development of monogenic forms of DM (Vaxillaire & Froguel, 2008). In this instance, candidate gene approach has identified multiple genes in the pathogenesis of maturity-onset diabetes of the young (MODY). Examples of such genes are the insulin

receptor substrate 1 (*IRS 1*), fatty acid binding protein (*FABP 2*) and glycogen synthase genes (Pozzilli, 2005). All of these genes can operate independently, or in tandem with one another to produce the final phenotype of impaired glucose metabolism and diabetes without any other contributing environmental factors.

However, the genetic aetiology of T2DM is polygenic and multi-component in nature (R. W. Grant, Moore, & Florez, 2009), involving complex gene-gene as well as gene-environmental or gene-dietary interactions (Qi, Hu, & Hu, 2008). Two genetic mechanisms exist that can confer risk to T2DM. The first mechanism is epigenetic modifications of specific segments of the DNA that alter how that segment is translated into functioning proteins. Epigenetics involves modifications around the DNA molecules in response to environmental stimuli, providing the body with a 'learned' response to such stimuli. The outcome is either repression or promotion of DNA segment translation into mRNA and subsequently modification of protein levels (Tremblay & Hamet, 2008). The other mechanism is single nucleotide polymorphisms in genes involved in pathways regulating insulin metabolism and functioning, modulating bodily response to glucose, as well as maintaining pancreatic beta-cells' viability.

In terms of timing, the epigenetic mechanism is a rapid modification of how the cells repress or promote certain genes, but persist mostly in the individual organism. On the other hand, modification via SNPs occurs more gradually and persists over generations. The focus of this dissertation is on how SNPs in certain genes predisposes to T2DM.

1.2. SINGLE NUCLEOTIDE POLYMORPHISMS

1.2.1. Overview

Single nucleotide polymorphisms (SNPs) are common variations in the human genetic code. SNPs are named as such because the variation seen is only on a single nucleotide/base pair basis (i.e. a C to T substitution). They occur at a rate of more than one percent, and differ from 'mutations' due to this higher prevalence. Due to this, SNPs have been implicated in the subtle variations seen between individuals of the common ethnic group or population. More importantly, as SNPs are part of the DNA segments that are transcribed into mRNAs and subsequently affect protein levels or structures, they have also been implicated in an individual's susceptibility to certain disease(s) and the varied response to xenobiotics.

There are two functional distinctions of SNPs: exonic and intronic. Exons are part of the mRNA that is ultimately translated into proteins. Introns are parts of the mRNA that are cleaved-off during pre-mRNA processing. Initially thought to be molecular 'trash', it has been shown that introns are functional components in the post-transcriptional modification of their parent mRNAs; they are collectively termed microRNAs or miRNAs (Muhonen & Holthofer, 2009). Where exonic SNPs could account for the alterations mentioned previously, intronic SNPs have an indirect way of imposing its actions by influencing mRNA processing post-transcription, by way of alteration of the sequence of the synthesised miRNAs.

As these SNPs alter the sequence of a gene, the messenger ribonucleic acid (mRNA) transcribed from the said gene would also be altered, leading to alterations in the expression level of the gene, or the sequencing of or halted production of its translated peptide. As a consequence, alterations in functional or structural protein structure and/or function could ensue from SNPs. This could mean modifications of

drug metabolising enzyme functions, defective actions of signalling peptides or instabilities in the collagenous framework of connective tissues.

1.2.2. SNPs and T2DM

The presence of a genetic factor in the aetiology of T2DM has been suspected after observations that i) the incidence of T2DM is higher in family members of patients, and ii) the concordance rate for T2DM between monozygotic twins is 55 – 100%. Furthermore, mutations in certain genes that is involved in glucose homeostasis, i.e. *KCNJ11* (potassium inwardly-rectifying channel, subfamily J, member 11) and *ABCC8* (adenosine triphosphate (ATP)-binding cassette, sub-family C, member 8) lead to rare forms of monogenic diabetes, as previously reviewed (Qi, 2008). These mutations suggest that the pathways of glucose or insulin metabolism can be altered at the molecular level via genetic modification; DM is the phenotypic outcome.

Traditionally, identification of genes affording risk for T2DM development is done using linkage analysis. A suspected gene is identified by way of its involvement in the pathway of glucose metabolism, and is then tested among first degree relatives of T2DM patients. As entailed, this approach has many disadvantages. The emergence of genome-wide association studies (GWAS) in mid-2000s has made the identification of genetic markers easier. In this approach, the whole genome is screened for potential variations between T2DM and non-diabetic individuals. This has led to the era of the SNPs.

Various candidate genes and their variations (by way of polymorphisms or mutations) have been implicated. Human peroxisome proliferator-activated receptor gamma (*PPARγ*) Pro12Ala polymorphism and *KCNJ11* Glu23Lys polymorphism are among the first that have been repeatedly shown in various populations to be common, and to be associated with T2DM risk. The *PPARγ* SNP provides protection for the

carrier against the development of T2DM ((Horiki et al., 2004; Scacchi et al., 2007)), whereas the opposite is true for the *KCNJ11* SNP ((Alsmadi, Al-Rubeaan, Wakil, et al., 2008; Thorsby et al., 2009)). There are currently 38 verified SNPs that are proven to affect T2DM risk (Petrie, Pearson, & Sutherland, 2011), of which several are outlined in Table 2.1.

The findings from these studies truly prove that genetics play an important role in T2DM development. Among the 38 susceptibility genes, the finding by Grant and his team when they established a relationship between variations in the *TCF7L2* gene and risk of T2DM (S. F. Grant et al., 2006) has reignited interest in the search for a genetic marker for this disease. As such, the invention of the GWAS has facilitated this purpose but to date, no other association has been found to be as strong as *TCF7L2's* in conferring risk to T2DM in multiple populations globally.

To date, there was only one previous work studying the effects of *TCF7L2* on the risk of T2DM in Malaysia. In this small cross-sectional study, it was found that *TCF7L2* did not influence the risk of T2DM in 165 Malaysian subjects (Vasudevan, Ismail, Ali, & Mansor, 2009). However, this study suffered from a small sample size (50 T2DM without hypertension, 55 T2DM with hypertension and 60 healthy subjects), an inconsistent method (hot start PCR with subsequent restriction fragment length polymorphism [RFLP] analysis) with no ethnic specific analysis. There were also several other studies looking at other SNPs and its influence on T2DM in Malaysia. SNPs in KCNQ1 were also found to associate with T2DM in Malaysian Malays (Saif-Ali, Muniandy, et al., 2011) and Chinese, with carriers of the protective haplotype shown to have a better index of β-cell function (Saif-Ali, Ismail, et al., 2011). A more recent study also suggested that variations in hepatocyte nuclear factor 4-α (HNF4α) associate with T2DM in a Malaysian population (Saif-Ali, Harun, Kamaruddin, Al-

Jassabi, & Ngah, 2012). As there is limited data on how SNPs in *TCF7L2* affect the prevalence of T2DM in a Malaysian population, the present study focused on this SNP.

Table 1.1: Chronology and details of several verified SNPs predisposing to T2DM

Year	Gene	Description	SNP (allele change, chromosome)	Phenotype		Ref
				Beta cell function	Insulin action	
2000	PPARγ	Peroxisome proliferator-activated receptor gamma	rs13081389 (A/G, 3)		Reduced	(Horiki, et al., 2004)
2003	KCNJ11	Potassium inwardly-rectifying channel, subfamily J, member 11	rs5215 (C/T, 11)	Reduced		(Alsmadi, Al-Rubeaan, Wakil, et al., 2008)
2006	*TCF7L2*	Transcription factor 7-like 2	rs7903146 (C/T, 10)	Reduced		(S. F. Grant, et al., 2006)
2007	CDKAL1	CDK5 regulatory subunit associated protein1-like 1	rs10440833 (A>T, 6)	Reduced		(Wu et al., 2008)
2007	HHEX/IDE	Haematopoietically expressed homeobox / insulin-degrading enzyme	rs5015480 (C>T, 10)	Reduced		(van Vliet-Ostaptchouk et al., 2008)
2007	SLC30A8	Solute carrier family 30 (zinc transporter), member 8	rs3802177 (C>T, 8)	Reduced		(Staiger et al., 2007)
2007	CDKN2A/B	Cyclin-dependent kinase inhibitor 2A/B	rs10965250 (A>G, 9)	Reduced		(Omori et al., 2008)
2007	IGF2BP2	Insulin-like growth factor 2 mRNA binding protein 2	rs1470579 (A>C, 3)	Reduced		(Maggie C. Y. Ng et al., 2008)
2007	FTO	Fat mass and obesity associated	rs11642841 (A>C, 16)		Reduced	(Sanghera et al., 2008)
2008	KCNQ1	Potassium voltage-gated channel, KQT-like subfamily, member 1	rs231362, rs163184 (A>C & G>T, 11)	Reduced		(Saif-Ali, Muniandy, et al., 2011; Unoki et al., 2008)

1.2.3. SNPs in TCF7L2 and T2DM

TCF7L2 is a nuclear transcription factor that is involved in a signalling pathway responsible for cellular homeostasis and development called the *wnt* pathway. Abnormalities in its signalling, as any variation in its components may cause, have been implicated in various developmental defects and human diseases such as cancer (Burwinkel et al., 2006; Folsom et al., 2008; M. S. Kim, Kim, Ahn, Yoo, & Lee, 2009). The gene encoding for *TCF7L2* is located on the long arm of chromosome 10 (S. F. Grant, et al., 2006).

Until the finding by Grant *et al*, the *wnt* pathway was never thought to be involved in glucose homeostasis; hence TCF7L2 was never contemplated as a candidate gene to be evaluated in T2DM. In the Icelandic population, healthy carriers of the genetic variants are 1.5 times more likely to develop T2DM in their lives as compared to those carrying the normal gene(S. F. Grant, et al., 2006). These correlations have been replicated using GWAS in various populations and ethnic groups across the globe (Chandak et al., 2007; Damcott et al., 2006; Groves et al., 2006; Humphries et al., 2006; Zhang et al., 2006). To date, there are several SNPs (rs7903146, rs12255372, rs7901695, rs11196205, rs4506565, rs7895340 and rs290487; rs, reference SNP ID) in this gene that have been shown to confer an increased risk to T2DM, depending on the ethnic groups examined. In the Caucasian and Indian population, rs7903146 and rs12255372 confer the highest risks (Chandak, et al., 2007; Florez et al., 2006), whereas in the Chinese population, it is rs290487 that confers the highest risk (Y.-C. Chang et al., 2007).

With regards to rs7903146, the allelic substitution is from C to T, as compared to rs12255372, in which the change is from G to T. As humans carry 2 copies of DNA, the possible combinations, called genotypes, are a mixture of both alleles. Therefore for rs7903146, the possible genotypes are CC (called wild-type), CT (heterozygous) and TT

(homozygous). In the case of rs12255372, the genotypes are GG, GT and TT (wild-type, heterozygous and homozygous, respectively). The effect of carrying the variant allele is seen in the heterozygous and homozygous groups, with increasing risk of developing T2DM in the homozygous genotype (due to more 'exposure' to the variant allele).

The influence of these genetic variations differs between ethnic groups. In the Caucasian and Indian ethnic groups, the frequency of the variant allele (minor allele frequency, MAF) for both rs7903146 and rs12255372 is high (20 to 30%). This differs with the Chinese and Japanese T2DM populations, in which the MAF for these SNPs are very low (2 to 6%) causing a blunting of their impact (Miyake et al., 2008; M. C. Y. Ng et al., 2007).

The SNPs at both loci of rs7903146 and rs12255372 occur in the intronic segment of the gene. As it is established that intronic SNPs do not alter the structure of the translated protein, presence of both SNPs do not have any effect on the structure of the *TCF7L2* protein. However, the level of expression of its mRNA in the SNP carriers is affected, with conflicting results reported in various tissue types. Whilst the expression level was found to be higher in pancreatic beta cells (Lyssenko et al., 2007) in carriers of rs7903146, the expression level in peripheral tissues such as muscle or adipose tissue is found to be either lower (Stéphane Cauchi et al., 2006) or the same (Elbein et al., 2007; Kovacs et al., 2008). Perhaps this suggests differing roles of *TCF7L2* in these tissues and as such, varying post-translational modifications of the mRNA.

A higher expression level of the *TCF7L2* protein has been shown to correlate with an increased expression of the insulin gene (Jin, 2008). Therefore it is still unclear how the polymorphisms lead to a reduction in glucose-stimulated insulin secretion. One possibility is that by measuring the total *TCF7L2* level in the pancreatic beta-cells, the

distinction between the different isoforms of *TCF7L2* could not be made. It has been shown that the parent *TCF7L2* protein is spliced differently according to different tissue types (Prokunina-Olsson, Kaplan, Schadt, & Collins, 2009; Prokunina-Olsson et al., 2009). It is possible that the increased levels noted in pancreatic beta cells are the inactive form of *TCF7L2* for that tissue type; however this has not been proven.

Presence of the variant T allele of rs7903146 has been implicated to cause a reduction in pancreatic beta cell function due to an impaired incretin effect (Lyssenko, et al., 2007; Schäfer et al., 2007). This was attributed to an impaired action of the GLP-1 peptide on the pancreatic beta cells, causing a blunted ability to stimulate early-phase insulin secretion. In an animal model, a lower level of the *TCF7L2* protein leads to a downregulation of receptors for GIP and GLP-1 on the pancreatic beta cells (Shu et al., 2009). However as the level of *TCF7L2* was reportedly either higher or lower in beta cells of carriers of rs7903146, the significance of this finding has yet to be concluded.

Another recent study also associated the T allele of the same SNP with increased gluconeogenesis by the liver in its carriers (Pilgaard et al., 2009). Gluconeogenesis is a normal physiological response by the liver parenchymal cells in response to low plasma glucose levels, i.e. hypoglycaemia. The process aims at alleviating hypoglycaemia by producing glucose from non-carbohydrate sources, such as lactate, glycerol and certain amino acids. Inappropriate gluconeogenesis has been postulated to be one of the mechanisms of hyperglycaemia in T2DM. Therefore, this observation in carriers of rs7903146 lends more weight to its role in increasing T2DM risk.

The combination of blunted incretin effect and increased glucose production leads to recurrent prolonged hyperglycaemia in healthy carriers of the variant *TCF7L2* gene, progressing eventually to T2DM over time. To the author's knowledge, the information on the variants of *TCF7L2* has not been well documented in the Malaysian population of Chinese, Indian and especially Malay ancestries.

1.3. PHARMACOGENOMICS OF ORAL ANTIDIABETIC AGENTS

1.3.1. Overview

Pharmacogenomics is a branch of pharmacology which studies the influence of genetic variations on the response of an individual to xenobiotics, which include pharmacological agents. It is a common observation that the same dosage of drug given to a group of patients of similar age, gender, ethnicity and physical attributes does not produce a uniform effect. Some would achieve the pharmacological benefit at a higher or lower dose, whereas others could develop serious adverse effects or be completely unchanged physiologically and/or biochemically. After the completion of the Human Genome project, it was discovered that these individual variations could be due to SNPs that occur quite regularly in the human genome.

The genetic variation could occur at various levels of drug-host interaction, such as variations in the cytochrome (CYP) P450 family of enzymes in the liver, or variations in the sulfonylurea receptor (SUR-1) on the pancreatic beta cells; to name a few (Becker et al., 2008; Nikolac et al., 2009). Both of these are due to genetic variations, i.e. SNPs of the DNA sequence encoding for the enzymes or the receptor. Whilst the effect of SNPs on warfarin metabolism and drugs metabolised by the cytochrome P450 family have been widely discussed (Wilffert et al., 2011), the impact of SNPs on antidiabetic medications are only recently being explored.

Treatment of T2DM is mainly targeted at normalising plasma glucose levels. There are a number of pharmacological agent classes to achieve this goal. Most T2DM patients are initially treated with metformin, a biguanide antidiabetic agent that promotes glucose utilisation in the peripheral tissues, i.e. skeletal muscle and lipocytes. This agent works well in the premise of a near-normal pancreatic beta cell capability of producing adequate amount of insulin. But as the disease progresses, reduction in beta

cell function would mean that usage of metformin alone is insufficient. A second group of antidiabetic agents is usually introduced at this point, which are sulfonylureas.

The sulfonylurea group of agents acts via the ATP-sensitive potassium channels on pancreatic beta cells to directly enhance insulin secretion. In Malaysia, the combination treatment of metformin and a sulfonylurea is the most preferred in T2DM patients as it is cost-effective and relatively safe. Subsequently, most patients with T2DM for more than a decade would ultimately require insulin therapy to maintain normoglycaemia. However, there remains a subset of patients who are newly-diagnosed but are found to be resistant to the aforementioned combination regime, requiring early insulin therapy. Various lifestyle factors could attribute to this, but the core problem is thought to be the pharmacogenomics of these agents in relation to the patients.

It has been well documented that the CYP2C9 enzyme metabolises the sulfonylurea antidiabetic agents, hence any variations of the same enzyme could accelerate or slow down their metabolism. This has the consequence of either poor normoglycaemic effect, or increased hypoglycaemic risk, respectively. Identifying patients who are carriers of the variants would ensure safety to those harbouring the genes at risk, by way of increasing or decreasing the dosage of sulfonylureas, or prescribing them a different type of antidiabetic agent altogether.

1.3.2. SNPs and the Pharmacogenomics of Antidiabetic Agents

Due to its interference with pancreatic beta cell function, variant *TCF7L2* in theory could affect agents that stimulate insulin secretion, such as the sulfonylurea group of drugs. A study in Scotland has shown that the presence of the variant T allele for both rs7903146 and rs12255372 leads to poorer therapeutic response with the sulfonylurea group of drugs in treatment-naïve patients (Ewan R. Pearson et al., 2007).

It was found that carriers of the variant *TCF7L2* are 2 times more unlikely to achieve satisfactory HbA_{1c} reduction after 12 months of treatment initiation.

Moreover, as carriers of the SNPs associate with an impaired entero-insular axis due to blunting of the incretin effect, response of the newer antidiabetic agents that utilises the incretin system such as the DPP-IV inhibitors (e.g. sitagliptin) and GLP-1 analogues (e.g. exenatide) could be affected. This has not been shown in any study thus far.

KCNJ11 polymorphism has been shown to affect response to sulfonylureas (Sesti et al., 2006) and repaglinide (He et al., 2008; Yu et al., 2010). Carriers of the E23K polymorphism of this gene has been shown to be more prone to secondary failure to sulfonylureas, most probably due to worsening of impairment of beta-cell function in its secretory capacity (Sesti, et al., 2006). Therefore, it was not surprising that the same polymorphism has been implicated in a lower risk for severe sulfonylurea-induced hypoglycaemia requiring emergency treatment (Holstein, Hahn, Stumvoll, & Kovacs, 2009).

Repaglinide belongs to the meglitinide group of antidiabetic drugs. Due to its rapid onset and short duration of action, it is taken before meals to stimulate postprandial insulin release similar to that found physiologically. Though structurally unrelated to the sulfonylurea group, the mechanism of action of repaglinide is similar in that it stimulates insulin release from beta-cells by binding to the sulfonylurea binding site. As such, the E23K polymorphism of KCNJ11 affected this mechanism, leading to a reduction in the potency of repaglinide in the SNP carriers. This is reflected via a higher HbA_{1c} and fasting plasma glucose (FPG) levels (He, et al., 2008).

As for *TCF7L2* variants, a study reported that the variant allele at rs7903146 conferred higher sensitivity to repaglinide, resulting in lower FPG and HbA_{1c} levels (Yu, et al., 2010). The reason for this remains elusive. As *TCF7L2* SNPs affect the

metabolism of glucose by way of reducing pancreatic beta cell function, in theory any agents modulating insulin release would be affected by these variations. However, the data is lacking in this area; the author aspires to fill this void in knowledge by the results of this work.

1.4. OBJECTIVES

1.4.1. Research Objectives

The objective of this study is to ascertain the relationships between single nucleotide polymorphisms in the *TCF7L2* gene with type 2 diabetes mellitus risk and glycaemic control in a Malaysian population, specifically:

1. To determine the allelic and genotypic frequencies of SNPs in the *TCF7L2* gene (rs7903146, rs12255372, rs7901695, rs11196205 and rs4506565) in a Malaysian population
2. To correlate between the presence of the SNPs to the risk of developing T2DM
3. To associate the effect of carrying the SNPs to clinical treatment outcome with oral antidiabetic agents and/or insulin

1.4.2. Hypotheses

The **null** hypotheses (H_0) of this study are:

1. There is no association between *TCF7L2* SNPs and risk of T2DM in a Malaysian population.
2. There is no effect of carrying the *TCF7L2* SNPs on glycaemic control with oral antidiabetic agents and/or insulin.

The **research** hypotheses (H_1) for this study are:

1. There is an association between *TCF7L2* SNPs and risk of T2DM in a Malaysian population.

2. There is an effect of carrying the *TCF7L2* SNPs on glycaemic control with oral antidiabetic agents and/or insulin.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Blood collection

i. BD Vacutainer® blood collection tubes, lithium heparin, 6.0 mls (BD, Franklin Lakes, New Jersey, USA)

ii. BD Vacutainer® Eclipse™ blood collection needles, various gauge sizes, (BD, Franklin Lakes, New Jersey, USA)

2.1.2. Leucocyte extraction

i. Red blood cell lysis buffer (iNtRON, Gyeonggi-do, Korea)

ii. Disposable plastic pipettes, 10 mls (local)

iii. Falcon tubes, 15 mls (BD, Franklin Lakes, New Jersey, USA)

2.1.3. Genomic DNA extraction

i. QIAamp DNA Blood Mini kit, 250 samples (Qiagen, Hilden, Germany). The components of this kit include the Qiagen Protease solution, lysis buffer AL solution, wash buffers AW1 and AW2 solutions, spin columns and collection tubes.

ii. Microcentrifuge tubes, 1.5 mls (Orange Scientific, Braine-l'Alleud, Belgium)

2.1.4. Real-Time Polymerase Chain Reaction (PCR)

i. TaqMan SNP genotyping assays, details in table 2.1 (Applied Biosystems, Carlsbad, California, USA)

ii. MicroAmp® Fast Reaction PCR Tubes with flat caps, 0.1 mls (Applied Biosystems, Carlsbad, California, USA)

iii. MicroAmp® Fast Optical 48-well Reaction Plates (Applied Biosystems, Carlsbad, California, USA)

2.1.5. Instrumentations

i. Biosafety cabinet class I

ii. Eppendorf Research® adjustable volume pipettes and corresponding tips (Eppendorf, Hamburg, Germany)

iii. Fine vortex machine

iv. Freezer, -20°C

v. Minispin Plus® centrifuge (Eppendorf, Hamburg, Germany)

vi. Refrigerated centrifuge

vii. StepOne Real-Time PCR machine (Applied Biosystems, Carlsbad, California, USA)

viii. Waterbath

2.1.6. Softwares

i. Haploview version 4.2 (Broad Institute of MIT and Harvard, Massachusetts, USA)

ii. IBM SPSS Statistics version 19 (IBM, New York, USA)

iii. OpenEpi (Rollins School of Public Health, Emory University, Georgia, USA)

iv. StepOneTM Software version 2.1 (Applied Biosystems, Carlsbad, California, USA)

Table 2.1: TaqMan SNP genotyping assay information

SNP	Assay ID	Amplified region
rs7903146	C__29347861_10	TAGAGAGCTAAGCACTTTTTAGATA[**C/T**] TATATAATTTAATTGCCGTATGAGG
rs12255372	C___291484_20	TGCCCAGGAATATCCAGGCAAGAAT[**G/T**] ACCATATTCTGATAATTACTCAGGC
rs4506565	C____384582_10	TCTCGGATATGGCGACCGAAGTGAT[**A/T**] TGGGGCCCTTGTCAAGGGTCTCTAT
rs11196205	C__27432600_10	GAAAGTTCTCAACATTTATAACTAC[**G/C**] AGCAGTATGTAAGAGAGTTATGGTT
rs7901695	C____384583_10	CATATAAATGGTATCATAAAATCTA[**T/C**] GGGCTTTTGTGTCTGTCTGCTTTCA

Letters in brackets [] are sites of polymorphism, with the corresponding allele substitutions

2.2. METHODS

2.2.1. Study Design and Recruitment of Subjects

A case-control study was set up. Cases were defined as type 2 diabetes mellitus patients from University of Malaya Medical Centre's (UMMC) pool of T2DM patients attending the primary care and specialist clinics. Their blood samples were obtained when they attended the Centralised Blood Collection Centre in UMMC for their blood test prior to their follow-up appointments. Criteria for inclusion and exclusion of patients are outlined in Table 2.2.

Non-diabetic control samples were obtained from two pools of patients. One pool is from healthy volunteers attending UMMC's Blood Bank mobile blood donation exercises around Klang Valley. The other pool of samples came from non-diabetic patients attending the Centralised Blood Collection Centre in UMMC to have their blood taken for routine testing prior to their follow-up appointments. The inclusion and exclusion criteria for the non-diabetics are listed in Table 2.3.

During the interview prior to signing of informed consent (Appendix 4), data such as personal medical history, family history and social history of both cases and controls were obtained (Appendix 5). Due to time constraints during the initial contact with the subjects, incomplete data was obtained through telephone interviews at a later date. All participants were given a copy of the study information sheet (Appendix 6) which briefly described the objective of the study, the protocols/procedures involved and contact information of the researchers.

Table 2.2: Inclusion and exclusion criteria for recruitment of T2DM patients

(a)	Inclusion Criteria
I.	Type 2 diabetes mellitus as diagnosed by a physician
II.	Belongs to any of the three major ethnic groups in Malaysia (Malay, Chinese or Indian) only
III.	Above 30 years of age
IV.	On any treatment regime for T2DM
V.	Able and willing to give written consent
(b)	Exclusion Criteria
I.	Type 1 diabetes mellitus
II.	Secondary diabetes mellitus, i.e. due to hepatic or biliary tree pathologies
III.	Mixed ethnic group parentage

Table 2.3: Inclusion and exclusion criteria for recruitment of healthy volunteers

(a)	Inclusion Criteria
I.	Not diagnosed with diabetes mellitus; or fasting plasma glucose levels not exceeding 6.5mmol/L, or a random plasma glucose levels not exceeding 11mmol/L
II.	Belongs to either of the three major ethnic groups in Malaysia (Malay, Chinese or Indian) only
III.	Above 30 years of age
IV.	Able and willing to give written consent
(b)	Exclusion Criteria
I.	Family history of T2DM
II.	Mixed ethnic parentage

2.2.2. Sample Collection

Six millilitres of blood were withdrawn into lithium heparin tubes (BD Vacutainer®) and stored on ice prior to the transport back to the laboratory. Samples from patients attending the Centralised Blood Collection Centre were obtained via phlebotomy of arm veins by the researcher during his attachment at the Centre, after written consent was signed. Samples from the healthy volunteers were obtained by nurses or medical assistants of UMMC's Blood Bank during the mobile blood donation exercises after written consent was signed.

2.2.3. Sample Preparation

Deoxyribonucleic acid (DNA) was extracted from leucocytes of the subjects. To extract the leucocytes from the whole blood, the samples were mixed with a red cell lysis buffer (iNTRON) at a ratio of 1:3 in separate falcon tubes, which were then centrifuged at 4000 rpm for 10 to 15 minutes. After centrifugation, the liquid lysate was discarded, leaving a solid leucocyte-rich pellet at the bottom of the falcon tubes. This step was sometimes repeated twice to ensure the purity of the leucocytes and to increase the amount of the leucocytes produced. This leucocyte pellet was then diluted in autoclaved nuclease-free water (200µl) and used for genomic DNA extraction.

2.2.4. Genomic DNA Extraction

The extraction was done using the DNA Blood Mini Kit (Qiagen) according to the protocol as provided by the manufacturer, as detailed below.

A mixture of 20µl Protease, 200µl leucocyte pellet and 200µl of buffer AL was made inside individually labeled 1.5ml microcentrifuge tubes. After mixing the

solutions and briefly centrifuging to remove droplets from the cap, the tubes were then incubated in a waterbath set at 56°C for 10 minutes. At the end of the 10 minutes, 200µl of ethanol (99%) was added to the tubes and mixed well with pulse vortex for 10 to 15 seconds. The final mixture was then transferred to the spin column in the 2ml collection tubes.

The spin columns were centrifuged at 8000rpm for one minute. The centrifugation speed and time was sometimes adjusted to higher levels and/or duration, ensuring that the spin column is completely empty at the end of this step. The filtrate collected in the collection tubes was discarded and the spin columns were transferred to a new set of collection tubes for the next step. Next, 500µl of buffer AW1 was added to the spin columns and centrifuged at 8000rpm for one minute. Again, this centrifugation step was sometimes adjusted to higher levels and/or duration, ensuring that the spin column is completely empty at the end of this step. The filtrate was discarded and the spin column transferred to a new set of collection tubes before 500µl of buffer AW2 is added to the columns. The columns were centrifuged at 14000rpm for 3 minutes or more, ensuring that the column is completely dry before proceeding to the final steps.

Discarding the filtrate from the previous step, the completely dry spin columns were transferred onto clean, individually labeled 1.5ml microcentrifuge tubes. A volume of 200µl of autoclaved nuclease-free water was added to the spin columns, and left to incubate at room temperature (25°C) for five minutes. After five minutes, the spin columns were centrifuged at 8000rpm for one minute, after which the spin columns were transferred to another set of clean, similarly labeled 1.5ml microcentrifuge tubes. A volume of 100µl autoclaved nuclease-free water was added to the spin columns and left to incubate for another three minutes. Following that, the spin colums were centrifuged at 8000rpm for one

minute, after which the spin columns were discarded. The eluted genomic DNA collected in the two sets of microcentrifuge tubes were stored in 100µl aliquots each at -20°C.

2.2.5. Clinical Data

During recruitment of cases, some data were obtained during consent signing. Incomplete and medication history, as well as biochemical parameters, were obtained by accessing the patients' medical records via UMMC's PTj Maklumat Pesakit (Centre of Patient Information). This was done on a periodical basis. The observed parameters are outlined in Table 2.4. All parameters were only recorded once after the subjects were seen and consent was obtained.

Table 2.4: Parameters observed during interviews and via medical records

	Parameter	Specific information
i.	Personal Medical History	• All medical illnesses • Medication history • Duration of T2DM • List of diabetic complications
ii.	Family History	• Parental ethnicity • History of T2DM
iii.	Anthropometric parameters	• Weight and height
iv.	Biochemical parameters	• Glycated haemoglobin fraction (HbA_{1c}) • Renal function profile • Liver function profile

2.2.6. Single nucleotide polymorphisms genotyping

(a) Overview

Five SNPs in the *TCF7L2* gene were chosen to be genotyped: rs7903146 (C>T), rs12255372 (G>T), rs4506565 (A>T), rs11196205 (G>C) and rs7901695 (T>C). These SNPs were chosen as their associations with T2DM were strongest in multiple populations examined (Chandak, et al., 2007; Horikoshi et al., 2007; Rees et al., 2008; Thorsby, et al., 2009; Tong et al., 2009). For this purpose, real-time polymerase chain reaction (PCR) was selected. Initially, attempts to recreate an experiment as observed in a journal paper (Bodhini, Radha, Dhar, Narayani, & Mohan, 2007) failed as the parameters described by the author could not be replicated (conventional PCR with restriction fragment length polymorphism; RFLP analysis of the product).

(b) Real-Time PCR

The disadvantage of conventional PCR is that after obtaining the desired PCR product, time consuming post-PCR procedures are still required to:

i) confirm the sequence of nucleotides of the product, and in the case of SNP genotyping/analysis,

ii) analyse the product for presence or absence of the SNPs (i.e. by way of RFLP). The invention of real-time PCR has significantly simplified these processes. The principle behind real-time PCR is the use of fluorescence detection during the PCR itself.

In real-time PCR, besides the normal components of PCR, there is an additional component of either a non-specific fluorescent dye which only intercalates with double-stranded DNA, or oligonucleotide sequences called probes which are labelled with a fluorescent reporter that is specific for the intended amplified

sequence. Whilst the non-specific dye will signal any presence of double-stranded DNA, the probes will only emit a fluorescent signal once it hybridised with complementary nucleotides on the amplified DNA segment.

Usage of real-time PCR in SNP genotyping has significantly reduced the time needed, thus improving throughput of the experiment. Furthermore, the specificity of the PCR product is very high, as fluorescence is only detected when both the primers and the probes anneal to the amplified sequence. Therefore, the researcher has selected this method for the purpose of SNP genotyping in this study.

Detection of the *TCF7L2* gene SNPs was done using the TaqMan SNP genotyping assays developed by Applied Biosystems (AB). Each assay contains the forward and reverse primers for the region containing the SNP, and two probes for each possible allele (i.e. wild-type or mutant allele) labeled with distinct fluorescent markers. The PCR and detection of fluorescence was done using the AB StepOne Real-Time PCR system.

The PCR reaction was prepared in individually labeled 0.1µl MicroAmp™ Fast Reaction PCR tubes (AB). A mastermix solution *sans* the genomic DNA was prepared by mixing the TaqMan SNP genotyping assay solution (20x) with the Genotyping Mastermix solution (10x) (AB) in a 1:10 ratio. Eight µl of this mastermix was then aliquoted into the aforementioned PCR tubes, before two µl of the genomic DNA added, making a final reaction volume of ten µl. The PCR tubes were then arranged on the MicroAmp™ Fast Optical 48-Well Reaction plates (AB) for easy loading into the StepOne Real-Time PCR system.

Before running the real-time PCR, the setup for the machine was done using the StepOne™ Software version 2.1 (AB). The setup includes specification of the

SNP genotyping assay used, the arrangement of individual samples on the plate and the selection of cycles with their appropriate temperatures. The genotyping started with a pre-PCR read at 60°C for 30 seconds, followed by a holding stage at 95°C for ten minutes. This is followed by 45 cycles of 95°C for 15 seconds and 60°C for one minute, ending with a post-PCR read at 60°C for 30 seconds. Genotypic distinction was made by analyzing the raw fluorescence data obtained from the machine using the same software.

The genotyping success rate was 99 – 100% as some DNA samples have degraded during storage leading to unsuccessful PCR on those samples.

2.2.7. Statistical Analysis

(a) Power and Sample Size Calculation

This was done using the OpenEpi program, an open-source software available online which was developed by the Rollins School of Public Health, Emory University, Georgia, USA. The number of samples needed for both groups was initially calculated using allelic frequency data from a journal paper (Chandak, et al., 2007) and was found to be 800 each, to obtain a power of 80%.

However, when ethnicity is considered, there were considerable discrepancies in terms of genotype distribution between the three major ethnic groups of Malay, Chinese and Indian. As data on genomic prevalence were accumulated, the sample size for the Malay ethnic group was recalculated to be 188 people in each case and control groups. A similar recalculation for the Chinese and Indian ethnic groups yielded sample sizes of 102 and 138 people, respectively. These numbers was to ensure a power of 80% for each ethnic group.

(b) Population Genetics

Genetic data obtained has to fulfill the Hardy-Weinberg equilibrium, to ensure that variations seen in the population are distributed normally, and is not due to interference from outside of said population. The Hardy-Weinberg model enables comparisons of a population's genetic structure over time with the expected genetic structure if the population is not evolving (i.e. in Hardy-Weinberg equilibrium). This calculation was done using the Haploview software, using the Chi-square test to determine statistical significance.

(c) Descriptive Statistics

Determination of mean, standard deviations, range, frequency and percentage distribution was done using the IBM SPSS Statistics software (version 19) descriptive statistic function.

(d) Association Testing and Comparison of Means

Allelic and genotypic association testing was done using OpenEpi software. This calculator uses the chi-square methodology in determining the *p*-value and the odds ratio with its 95% confidence interval. Continuous data obtained was first analysed for normality of distribution before an appropriate test was selected to analyse it. Normally distributed data was analysed using the independent sample t-test or the one-way ANOVA, whereas data that was not normally distributed was analysed using the Mann-Whitney test.

(e) Haplotype and Linkage Analysis

Linkage analysis was done to observe any interactions between the five SNPs of *TCF7L2* genotyped. A SNP is said to be in linkage disequilibrium (LD) with

another SNP if the variant alleles of both SNPs are carried together by the subject. Haplotype is the acronym for haploid genotype, where a combination of alleles at different loci of the same gene, i.e. the five SNPs of *TCF7L2*, are compared against one another and against the outcome measured or the disease state observed. Linkage and haplotype analyses were done using the Haploview software in order to detect interaction between the SNPs in predisposing T2DM.

CHAPTER 3

RESULTS

3.1. BASIC CHARACTERISTICS OF STUDY SUBJECTS

3.1.1. Distribution of continuous data

The distributions of all continuous data were near normal, as reflected in Figure 3.1. Therefore, all continuous data were analysed for significant difference between groups using parametric tests.

Figure 3.1: Data regarding normality of distribution of continuous variables in study subjects, with corresponding histograms

(i) Age

Skewness	Std. Error of Skewness	Kurtosis	Std. Error of Kurtosis	Mean	Median
-0.755	0.077	0.737	0.154	55.39	56.00

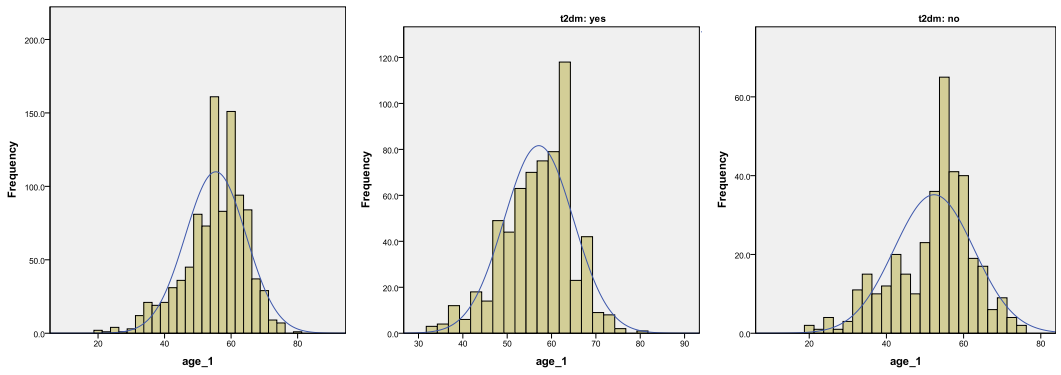


Figure 3.1 (continued)

(ii) Weight and height

Skewness	Std. Error of Skewness	Kurtosis	Std. Error of Kurtosis	Mean	Median
Weight					
1.779	0.078	8.324	0.155	68.711	66.000
Height					
-0.664	0.078	2.948	0.155	1.581	1.575

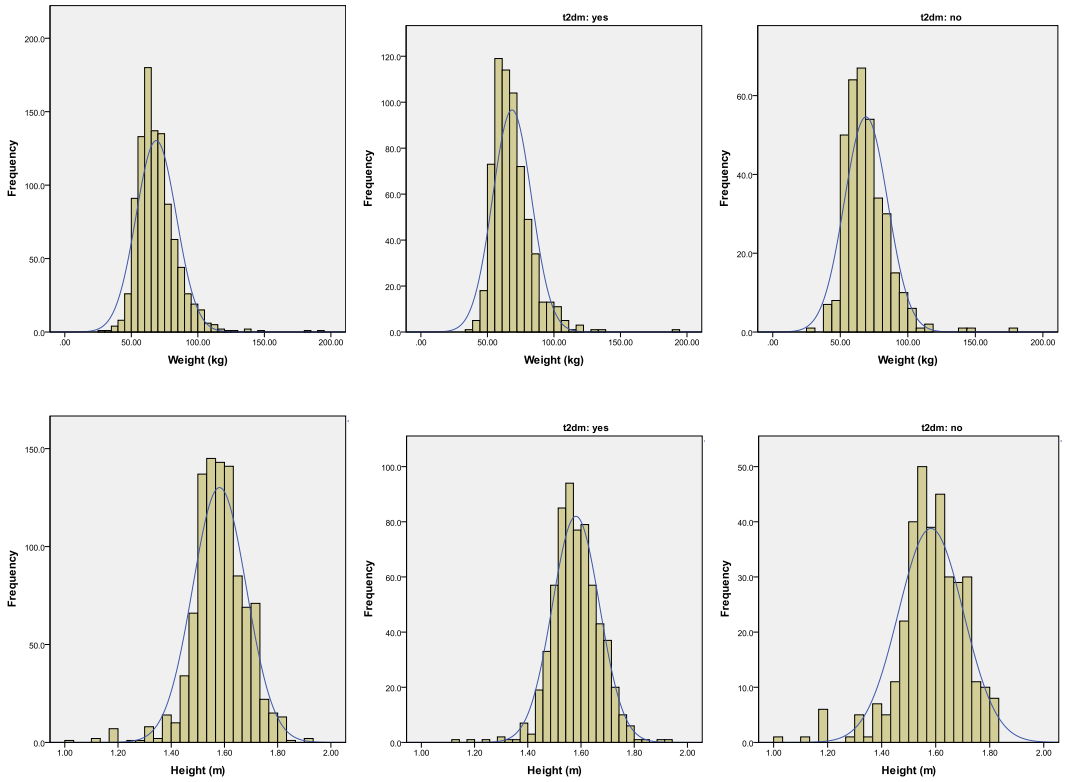
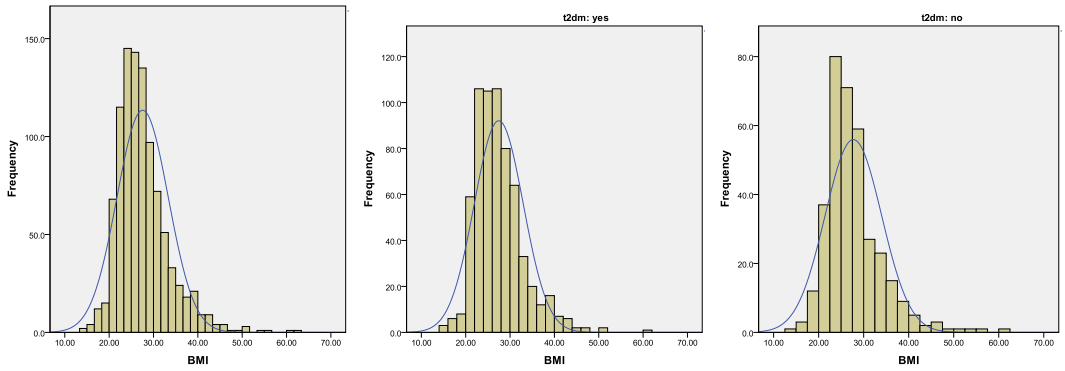


Figure 3.1 (continued)

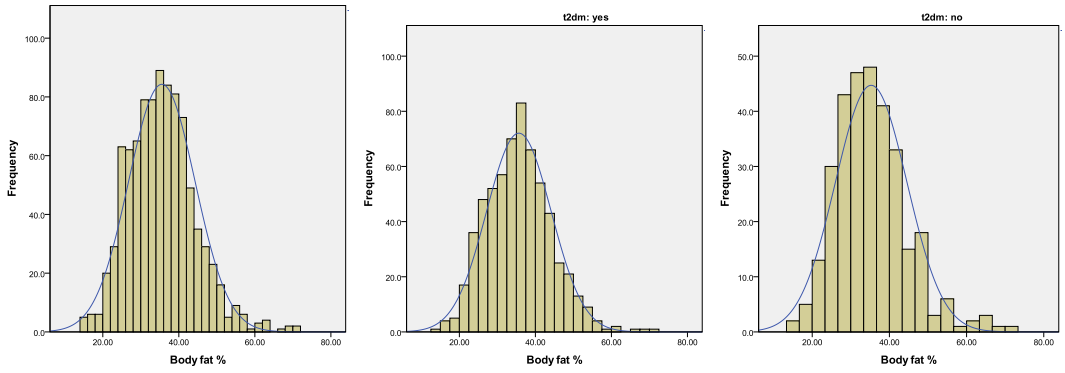
(iii) Body-mass index

Skewness	Std. Error of Skewness	Kurtosis	Std. Error of Kurtosis	Mean	Median
1.432	0.078	3.998	0.155	27.547	26.481



(iv) Body fat percentage (BF%)

Skewness	Std. Error of Skewness	Kurtosis	Std. Error of Kurtosis	Mean	Median
0.594	0.080	0.905	0.161	35.527	35.053



3.1.2. Overview

The basic characteristics of the study subjects are described in detail in Table 3.1, in which significant differences between the cases (T2DM patients) and controls (non-T2DM subjects) are highlighted. The differences of the basic characteristics between the three ethnic groups are also summarised in the same table. A total of 1008 subjects were recruited into this research project.

In general, the cases were older than controls (difference of 3.6 years, $p < 0.001$). This was reflected in all three ethnicities, with the difference in the Chinese subjects the greatest (6.3 years, $p=0.01$). The age difference observed in the Indian was not significant. According to age groups, a bigger proportion of the cases exceeded the age of 55 years compared to the controls ($p < 0.001$). This pattern was replicated in the Malay and Chinese subjects, but not in the Indians.

The body-mass index (BMI) of the overall subjects did not differ significantly when compared between the cases and controls. There was no difference in the BMI of cases and controls in the Malay and Indian ethnic groups. However, the Chinese controls had significantly higher BMI than their case counterparts (difference of 2.5kg/m^2, $p=0.01$). In the overall data for males, there was a significantly higher BMI in the controls compared to the cases (difference of 1.3 kg/m^2, $p=0.04$). This pattern is replicated in the Chinese males (difference of 6.1 kg/m^2, $p=0.01$). However, the Malay and Indian male subjects had no significant difference in BMI. In the female subjects, there was no significant BMI difference between the cases and controls.

The majority of subjects were deemed overweight in both groups, disregarding BMI cut-offs (>25kg/m^2 and >23kg/m^2 for Caucasians and Asians, respectively). Taking the cut-off for Asians, there were a slightly higher proportion of overweight cases compared to controls, albeit statistically insignificant. This pattern was reflected

in the ethnic groups involved, with the exception being the Chinese ethnic group in which the proportions were reversed (p=0.004).

Body fat percentage (BF%) was calculated using the Deurenberg equation as detailed in the footnote of Table 3.1. In general, the BF% of cases was higher than controls. However in Chinese subjects, the controls have a higher BF% compared to the cases, which reflected the observations regarding their BMI previously. These observations were not statistically significant. Excess body fat is described as having a BF% of more than 25% in males or 33% in females. Using this categorisation, it was observed that a bigger proportion of the cases had excess body fat compared to controls, with the exception of the Chinese subjects in which the reverse was observed. Again, these observations were not statistically significant.

When the cases were compared across the three ethnic groups, significant difference was observed in the mean age (Chinese highest, p <0.001), proportion aged more than 55 years (Chinese biggest, p=0.009), overall mean BMI (Indian highest, p=0.004) and mean BMI in males (Indian highest, p=0.004). All other observations in cases across the ethnicities were not significantly different.

Table 3.1: Basic characteristics of study subjects

Variable	Overall			Malay			Chinese			Indian		
	T2DM	*Non-T2DM*	*p-value*	*T2DM*	*Non-T2DM*	*p-value*	*T2DM*	*Non-T2DM*	*p-value*	*T2DM*	*Non-T2DM*	*p-value*
n (%)	642 (63.7)	366 (36.3)		390 (59.5)	265 (40.5)		111 (63.1)	65 (36.9)		141 (79.7)	36 (20.3)	
Age (years ± SD)[a]	57.0±8.0	53.4±10.5	**<0.001**	56.2±7.4	53.3±10.0	**<0.001**	60.0±8.5	53.7±12.9	**0.01**	57.1±8.6	53.1±12.0	0.13
Age group distribution (%)[b]												
<55 years	33.3	51.1	**<0.001**	38.2	53.6	**<0.001**	17.4	55.4	**<0.001**	31.9	25.0	0.42
55 years or >	66.7	48.9		61.8	46.4		82.6	44.6		68.1	75.0	
Male proportion (%)[c]	46.4	50.6	0.24	44.6	47.8	0.42	50	65.7	0.12	49.2	55.6	0.57
BMI overall (kg/m^2±SD)[a]	27.5±5.5	27.7±6.3	0.51	27.7±5.7	27.5±5.8	0.62	25.9±5.3	28.4±6.8	**0.01**	28.0	28.1	0.91
BMI male (kg/m^2±SD)[a]	27.3±5.8	28.6±6.7	**0.04**	27.7±6.0	28.1±5.5	0.52	25.0±5.5	31.1±9.1	**0.01**	28.3±4.7	28.9±8.7	0.69
BMI female (kg/m^2±SD)[a]	27.6±5.3	27.6±6.2	1.0	27.7±5.5	27.4±6.1	0.59	27.0±5.0	30.2±6.4	0.13	27.5±5.0	27.1±7.6	0.78
BMI>25 (%)[c]	64.4	62.6	0.58	66.1	62.3	0.35	48.6	62.9	0.08	71.9	63.9	0.41
BMI>23 (%)[c]	80.3	82.4	0.45	82.6	81.1	0.68	66.1	87.1	**0.004**	84.9	83.3	0.80
Body fat %* (±SD)[a]	35.6±8.5	35.3±9.3	0.55	36.0±8.6	35.0±8.8	0.20	34.2±9.0	37.7±10.7	0.10	36.0±7.6	34.8±10.9	0.56
Excess body fat** (%)[c]	86.2	83.0	0.21	87.6	81.9	0.06	78.8	90.6	0.19	87.9	83.3	0.58

*Calculated using Deurenberg equation, i.e. Body fat % =[1.2(BMI)+0.23(Age)-10.8(gender, male=1, female=0)-5.4; **Defined as body fat % >25 in males, >33 in females. Tested for significance using: [a], Independent samples T-test; [b], Spearman correlation analysis; [c], Chi-square test. **Bold** values are significant.

3.2. ASSOCIATION BETWEEN *TCF7L2* SNPS AND T2DM

3.2.1. Basic characteristics according to genotype

Table 3.2 describes the basic characteristics of the cases and controls when compared according to genotypes. In Table 3.2(i), characteristics of genotype carriers of rs7903146 were examined. Although differences existed between the genotype carriers within each group of subjects (cases or controls), the differences observed were not statistically significant. However when each genotype was compared between cases and controls, statistically significant differences were observed for mean age and proportion of subjects aged more than 55 years. Among carriers of the CC genotype, subjects with T2DM were significantly older than the controls, with a significantly bigger proportion of the cases aged more than 55 years. A similar observation was made among the CT carriers. However, the TT genotype did not show significant difference in terms of age between cases and controls.

Tables 3.2(ii)-(v) described the characteristics of genotype carriers of rs12255372, rs11196205, rs7901695 and rs4506565, respectively. The differences observed in rs7903146 are repeated in all these SNPs, with the differences in mean age and proportion of subjects aged more than 55 years being significant as well. In addition, for rs4506565, a significantly bigger proportion of TT cases have excess body fat compared to the TT controls ($p=0.05$).

Table 3.2: Basic characteristics of subjects in the T2DM and non-T2DM groups based on genotypes

(i) rs7903146

Groups	T2DM				Non-T2DM				T2DM vs non-T2DM p-value (CC/CT/TT)
Genotype	*CC*	*CT*	*TT*	p-value within group	*CC*	*CT*	*TT*	p-value within group	
n	494	131	15		318	43	5		
Male proportion (%)[a]	47.3	45.6	30.8	0.49	51.3	45.2	60.0	0.70	0.29/1.0/0.33[a]
Mean age (years±SD)[b]	**57.2±7.8**	**57.1±8.2**	55.2±6.4	0.62	**52.4±10.3**	**52.2±11.2**	48.6±9.9	0.71	**<0.001/0.01**/0.22[b]
Proportion >55 years (%)[c]	**66.7**	**67.7**	60.0	0.99	**49.1**	**46.5**	60.0	0.90	**<0.001/0.01**/1.0[c]
Mean BMI (kg/m^2±SD)[b]	27.4±5.6	27.7±5.4	27.3±3.2	0.85	27.5±6.0	28.3±7.2	33.4±12.5	0.09	0.74/0.62/0.34[b]
Proportion overweight* (%)[c]	79.3	82.9	86.7	0.92	82.3	83.3	80.0	0.27	0.31/1.0/1.0[c]
Mean BF% (%±SD)[b]	35.5±8.7	35.9±7.7	37.3±7.1	0.70	35.1±9.1	36.2±9.3	39.4±18.7	0.46	0.50/0.85/0.82[b]
Proportion with excess fat (%)**[c]	85.7	87.1	92.3	0.55	83.8	80.5	60.0	0.33	0.49/0.30/0.11[c]

*Overweight defined as BMI ≥23kg/m2; **Excess fat defined as BF% >25 in men or >33 in women

Tested for significance using: [a] Chi-square test; [b] ANOVA; [c] Spearman correlation analysis; **bold** values are significant

(ii) rs12255372

Groups	T2DM				Non-T2DM				T2DM vs non-T2DM p-value (CC/CT/TT)
Genotype	*GG*	*GT*	*TT*	p-value within group	*GG*	*GT*	*TT*	p-value within group	
n	520	108	12		330	30	5		
Male proportion (%)[a]	83.6	14.7	1.7	0.9	89.0	9.1	1.8	0.45	0.56/0.4/0.62
Mean age (years±SD)[b]	**57.0±7.8**	**58.0±7.7**	54.3±7.3	0.22	**52.4±10.3**	**52.8±11.4**	48.6±9.9	0.71	**<0.001**/**0.02**/0.29
Proportion >55 years (%)[c]	**65.9**	**72.9**	50.0	0.49	**48.2**	**53.3**	60.0	0.88	**<0.001/0.04**/0.73
Mean BMI (kg/m^2±SD)[b]	27.4±5.5	27.7±5.8	26.8±3.0	0.83	27.5±6.0	29.0±7.5	33.4±12.5	0.06	0.85/0.39/0.30
Proportion overweight* (%)[c]	80.0	81.1	83.3	0.72	81.7	89.7	80.0	0.35	0.53/0.28/0.88
Mean BF% (%±SD)[b]	35.4±8.6	36.6±8.1	34.7±6.3	0.39	35.1±9.0	36.8±10.4	39.4±18.7	0.4	0.58/0.97/0.61
Proportion with excess fat (%)[c]**	85.8	87.1	91.7	0.59	83.0	85.7	60.0	0.82	0.31/0.85/0.13

*Overweight defined as BMI ≥23kg/m2; **Excess fat defined as BF% >25 in men or >33 in women

Tested for significance using: [a] Chi-square test; [b] ANOVA; [c] Spearman correlation analysis; **bold** values are significant

(iii) rs11196205

Groups	T2DM				Non-T2DM				T2DM vs non-T2DM p-value (CC/CT/TT)
Genotype	*CC*	*CG*	*GG*	p-value within group	*CC*	*CG*	*GG*	p-value within group	
n	465	145	31		298	58	10		
Male proportion (%)[a]	47.8	44.2	37.0	0.46	51.1	48.1	50.0	0.92	0.22/0.37/0.37
Mean age (years±SD)[b]	**57.0±7.9**	**57.5±7.3**	**57.0±8.9**	0.84	**52.4±10.5**	**53.1±9.9**	**47.8±10.3**	0.33	**<0.001/0.003/0.03**
Proportion >55 years (%)[c]	**65.7**	**69.7**	66.7	0.44	**48.0**	**55.2**	40.0	0.50	**<0.001/0.05**/0.14
Mean BMI (kg/m^2±SD)[b]	27.4±5.6	27.6±5.4	27.4±4.6	0.95	27.7±6.0	27.3±6.8	30.1±9.9	0.47	0.5/0.77/0.46
Proportion overweight* (%)[c]	79.5	82.5	80.0	0.5	83.6	77.2	77.8	0.23	0.17/0.39/0.89
Mean BF% (%±SD)[b]	35.5±8.6	36.2±8.4	35.8±6.8	0.65	35.3±9.0	34.7±9.7	37.3±14.1	0.74	0.85/0.33/0.77
Proportion with excess fat (%)[c]**	86.0	86.0	88.9	0.84	84.3	79.2	66.7	0.17	0.55/0.25/0.13

*Overweight defined as BMI ≥23kg/m2; **Excess fat defined as BF% >25 in men or >33 in women

Tested for significance using: [a] Chi-square test; [b] ANOVA; [c] Spearman correlation analysis; **bold** values are significant

(iv) rs7901695

Groups	T2DM				Non-T2DM				T2DM vs non-T2DM p-value (CC/CT/TT)
Genotype	*TT*	*TC*	*CC*	p-value within group	*TT*	*TC*	*CC*	p-value within group	
n	482	113	15		307	37	5		
Male proportion (%)[a]	47.3	45.9	30.8	0.49	50.4	50.0	60.0	0.91	0.44/0.70/0.33
Mean age (years±SD)[b]	**57.1±7.8**	**57.3±8.1**	55.2±6.4	0.63	**52.4±10.3**	**51.1±10.7**	48.6±9.9	0.57	**<0.001/0.002**/0.22
Proportion >55 years (%)[c]	**66.1**	**67.0**	60.0	0.97	**48.5**	**43.2**	60.0	0.71	**<0.001/0.01**/1.0
Mean BMI (kg/m^2±SD)[b]	27.5±5.6	28.0±5.4	27.3±3.2	0.69	27.5±5.9	28.1±6.0	33.4±12.5	0.08	0.91/0.93/0.34
Proportion overweight* (%)[c]	80.0	84.7	86.7	0.21	82.3	86.1	80.0	0.64	0.43/0.84/0.74
Mean BF% (%±SD)[b]	35.6±8.7	36.4±8.1	37.3±7.1	0.55	35.1±9.0	35.3±8.9	39.4±18.7	0.57	0.43/0.5/0.82
Proportion with excess fat (%)**[c]	86.4	87.0	92.3	0.71	83.5	82.9	60.0	0.55	0.28/0.54/0.11

*Overweight defined as BMI ≥23kg/m2; **Excess fat defined as BF% >25 in men or >33 in women

Tested for significance using: [a] Chi-square test; [b] ANOVA; [c] Spearman correlation analysis; **bold** values are significant

(v) rs4506565

Groups	T2DM				Non-T2DM				T2DM vs non-T2DM p-value (CC/CT/TT)
Genotype	*AA*	*AT*	*TT*	p-value within group	*AA*	*AT*	*TT*	p-value within group	
n	495	125	20		308	37	5		
Male proportion (%)[a]	47.4	45.4	33.3	0.48	50.4	51.4	60.0	0.91	0.45/0.57/0.34
Mean age (years±SD)[b]	**57.2±7.8**	**57.2±8.1**	55.8±6.4	0.73	**52.4±10.3**	**51.2±10.7**	48.6±9.9	0.6	**<0.001/0.003**/0.19
Proportion >55 years (%)[c]	**66.6**	**68.5**	60.0	0.92	**48.7**	**43.2**	60.0	0.7	**<0.001/0.005**/1.0
Mean BMI (kg/m^2±SD)[b]	27.4±5.6	27.8±5.5	27.2±3.2	0.78	27.5±5.9	27.7±5.9	33.4±12.5	0.09	0.83/0.97/0.33
Proportion overweight* (%)[c]	79.3	82.9	85.0	0.3	82.7	83.3	80.0	0.98	0.24/0.96/0.8
Mean BF% (%±SD)[b]	35.5±8.7	36.0±7.9	36.8±6.7	0.71	35.1±9.0	34.9±8.8	39.4±18.7	0.57	0.55/0.49/0.77
Proportion with excess fat (%)[c]**	85.7	86.4	**94.4**	0.49	83.6	82.4	**60.0**	0.55	0.45/0.55/**0.05**

*Overweight defined as BMI ≥23kg/m2; **Excess fat defined as BF% >25 in men or >33 in women

Tested for significance using: [a] Chi-square test; [b] ANOVA; [c] Spearman correlation analysis; **bold** values are significant

3.2.2. Allele and genotype frequency in association with type 2 diabetes mellitus

Association between the SNPs observed with risk for T2DM is described in Table 3.3(i)-(v).

(i) rs7903146

Overall, the frequency of the minor (T) allele for rs7903146 was significantly higher in cases compared to controls (p=0.004). Each T allele carries an increase of 1.68 times the risk of T2DM compared to the C allele.

In a co-dominant model, each variant genotype was compared to the wildtype. The frequency of the heterozygous genotype (CT) was higher in cases compared to controls, with odds ratio (OR) of having T2DM in CT carriers at 1.96 times compared to wildtype (CC) carriers. This effect is still seen after correcting for age, gender and BMI (OR 1.72, 1.17 – 2.55). As for the homozygous variant genotype (TT), due to the low frequency of this genotype, the difference observed did not reach statistical significance.

A dominant model compares the CC genotype to the variant genotypes that contain the T allele (CT and TT). For this SNP, dominant modelling further strengthened the association observed with the CT genotype as evidenced by the smaller p-values (both the crude and adjusted).

When the ethnic groups were examined separately, the minor allele frequency was still noted to be higher in the Malay cases compared to controls. However this did not reach statistical significance. Similarly, more cases carry the variant genotypes (CT and TT) compared to controls but this observation was also not significant. The same pattern of observation was repeated when a dominant model was adopted for the Malay subjects. Adjustment with age, gender and BMI did not alter the association observed.

In the Chinese, fewer cases carried the variant allele compared to the controls. In particular, a significant difference in CT frequency was observed between cases and

controls, with a bigger proportion of the latter carrying CT compared to the former (adjusted p=0.03). Carrying the CT genotype conferred less risk of developing T2DM compared to the CC in the Chinese (OR 0.22, 0.06 – 0.83). There was a notable absence of carriers of TT genotype in this ethnic group.

In the Indian ethnic group, there were a higher proportion of cases carrying the CT genotype compared to controls. The significance of this difference was apparent after adjustment for age, gender and BMI was made (p=0.03). Carriers of CT are 2.43 times more likely to have T2DM compared to CC. This association was also replicated in the dominant model, where CT+TT carriers are 2.33 times more likely to have T2DM compared to CC (p=0.03).

(ii) rs12255372

For this SNP, the observation in the overall analysis mirrors that of rs7903146. The minor allele (T), heterozygous genotype (GT) and combined variant genotypes (GT+TT) frequencies were all significantly higher in the cases compared to controls. Even after adjustment, the significant association of GT and GT+TT genotypes with T2DM persisted.

In the Malays, although the T allele and GT genotype were more prevalent in the cases compared to controls, the difference observed is quite small, and did not lead to any association with T2DM. This is also the case when using the dominant model. Adjustment with age, gender and BMI further weakened the association.

As for the Chinese ethnic group, the frequencies of the T allele and GT genotype did not differ between the cases and controls. Furthermore as observed in rs7903146 previously, there seemed to be an absence of TT genotype carriers in the Chinese ethnic group. Examination of the dominant model also did not reveal any association with T2DM.

In the Indians, although there was no significant difference in the T frequency, the GT carriers are more prevalent in the cases compared to controls (p=0.04) and is associated with 2.39 times more risk of having T2DM. This association was further strengthened after adjustment for age, gender and BMI was made (OR 2.54, 1.07 – 6.04; p=0.03). Analysis of the dominant model (GT+TT) revealed a similar association when adjustment was made (OR 2.32, 1.05, 5.14; p=0.04).

(iii) rs11196205

The presence of the G allele of this SNP conferred 1.62 times more risk per allele of having T2DM compared to the C allele (p=0.0009). At the genotype level, carriers of CG were more prevalent in the cases compared to controls (p=0.006), which indicate that they were 1.6 times more likely to have T2DM compared to their CC counterparts. However, the significance of the association diminished after adjustment with age, gender and BMI was made. Ethnic-specific analysis of this SNP did not reveal any significant interaction between the G allele and variant genotypes (CG and/or GG) with T2DM.

(iv) rs7901695

In this SNP, the minor allele (C) frequency was higher in the cases compared to controls (p=0.007), with each C allele increasing the risk of having T2DM by 1.71 times. TC carriers were more prevalent in the cases. After adjustment for age, gender and BMI, they were found to have 1.73 times more risk of having T2DM compared to their TT counterparts. When a dominant model analysis was performed, carriers of the variant genotypes were 1.72 times more likely to have T2DM compared to their TT counterparts (p=0.007).

In the Malays, there were minimal differences in C allele, TC and CC genotype frequencies between cases and controls. The insignificant observations were made weaker after adjusted for age, gender and BMI.

As for the Chinese group, the frequency of the C allele was significantly lower in the cases compared to the controls (p=0.04). Each C allele carried conferred 5.3 times less risk of having T2DM. A similar pattern of distribution was observed for TC genotype, with carriers of TC 5.3 times less likely to have T2DM (adjusted p=0.02). Since there was an absence of CC carriers, dominant modelling did not alter this association.

The C allele was significantly more prevalent in Indian cases compared to their control counterparts (OR 3.15, 1.05 – 9.43; p=0.05). Furthermore, a very strong association was observed between TC carriers and T2DM (OR 4.8, 1.59 – 14.44; p=0.01). Adjustment for age, gender and BMI further strengthened the observation that TC carriers were 4.8 times more likely to have T2DM compared to TT carriers (p=0.005). In the dominant model after similar adjustment, the association persisted (OR 3.77, 1.45 – 9.79; p=0.007).

(v) rs4506565

Overall, there was a higher prevalence of the T allele in the cases compared to controls (p=0.0005), with each T carried conferring 1.97 times more risk of having T2DM. As for the genotypes, AT genotype carriers were 2.1 times more likely to have T2DM (p=0.0002). After adjusting for age, gender and BMI, the strength of the association was slightly diminished (OR 1.92, 1.26 – 2.91; p=0.002) but the significance persisted. Similarly, dominant modelling revealed that carriers of the variant genotypes were 1.95 times more likely to have T2DM post-adjustment (p=0.001).

In the Malay ethnic group, there were no significant differences observed between the cases and controls. This holds true even in the dominant model analysis.

In the Chinese group, a lower prevalence of the T allele was observed in the cases compared to controls (p=0.24). After adjusting for age, gender and BMI, there was a protective effect associated with the AT genotype in this ethnic group (OR 0.17, 0.03 –

0.87; p=0.03). This effect persisted when dominant modelling was used (OR 0.22, 0.05 – 1.0; p=0.05).

As for the Indians, the T allele was significantly more prevalent in the cases compared to controls (p=0.02). Each T allele carried increased the risk of having T2DM by 3.83 times. There were more AT carriers in the cases compared to controls, with an OR of 5.8 (1.97 – 17.11) after adjusting for age, gender and BMI (p=0.001). Using the dominant model, the association was still significant and strong (OR 4.61, 1.82 – 11.72; p=0.001).

Table 3.3: Allele and genotype frequencies of *TCF7L2* SNPs and association with T2DM

(i) rs7903146

rs7903146 C>T	T2DM	Non-T2DM	OR (95% CI)[a]	p-value[a]	Adjusted OR (95% CI)[b]	Adjusted p-value[b]
OVERALL						
T allele	0.12	0.08	**1.68[c] (1.18, 2.40)**	**0.004[c]**		
CC	0.78	0.86	*Reference*			
CT	0.20	0.13	**1.96 (1.35, 2.85)**	**3.9e-4**	**1.72 (1.17, 2.55)**	**0.006**
TT	0.02	0.01	1.93 (0.70, 5.37)	0.21	1.71 (0.59, 4.94)	0.32
CT+TT	0.22	0.14	**1.96 (1.37, 2.79)**	**2.1e-4**	**1.72 (1.19, 2.50)**	**0.004**
MALAY						
T allele	0.08	0.06	1.33[c] (0.83, 2.15)	0.29[c]		
CC	0.85	0.89	*Reference*			
CT	0.14	0.10	1.61 (0.97, 2.65)	0.06	1.64 (0.97, 2.77)	0.07
TT	0.01	0.01	0.72 (0.10, 5.13)	0.74	0.79 (0.11, 5.73)	0.79
CT+TT	0.15	0.11	1.54 (0.95, 2.50)	0.08	1.57 (0.94, 2.62)	0.08
CHINESE						
T allele	0.02	0.09	0.24[c] (0.06, 1.00)	**0.05[c]**		
CC	0.95	0.81	*Reference*			
CT	0.05	0.19	0.56 (0.17, 1.82)	0.34	**0.22 (0.06, 0.83)**	**0.03**
TT	Nil	Nil	NA	NA	NA	NA
CT+TT	0.05	0.19	0.56 (0.17, 1.82)	0.34	**0.22 (0.06, 0.83)**	**0.03**
INDIAN						
T allele	0.35	0.24	1.72[c] (0.83,3.55)	0.20[c]		
CC	0.39	0.57	Reference			
CT	0.52	0.39	2.12 (0.96, 4.67)	0.06	**2.43 (1.07, 5.50)**	**0.03**
TT	0.09	0.04	1.60 (0.41, 6.17)	0.50	1.88 (0.46, 7.77)	0.38
CT+TT	0.62	0.44	2.01 (0.96, 4.24)	0.07	**2.33 (1.07, 5.05)**	**0.03**

[a] Obtained using logistic regression analysis, unless otherwise stated; [b] Adjusted for age, sex and BMI; [c] Obtained using Chi-square test

(ii) rs12255372

rs12255372 G>T	T2DM	Non-T2DM	OR (95% CI)	p-value[a]	Adjusted OR (95% CI)[b]	Adjusted p-value
OVERALL						
T allele	0.10	0.06	**1.94 (1.29, 2.91)**[c]	**0.002**[c]		
GG	0.81	0.90	*Reference*			
GT	0.17	0.09	**2.29 (1.49, 3.50)**	**1.5e-4**	**1.95 (1.24, 3.05)**	**0.004**
TT	0.02	0.01	1.52 (0.53, 4.36)	0.43	1.57 (0.54, 4.59)	0.41
GT+TT	0.19	0.10	**2.18 (1.46, 3.25)**	**1.4e-4**	**1.89 (1.24, 2.88)**	**0.003**
MALAY						
T allele	0.06	0.05	1.33 (0.78, 2.25)[c]	0.36[c]		
GG	0.88	0.91	*Reference*			
GT	0.11	0.08	1.55 (0.89, 2.69)	0.12	1.52 (0.85, 2.72)	0.16
TT	0.01	0.01	0.70 (0.10, 5.03)	0.73	0.77 (0.11, 5.58)	0.8
GT+TT	0.12	0.09	1.47 (0.86, 2.51)	0.16	1.45 (0.83, 2.53)	0.2
CHINESE						
T allele	0.01	0.02	0.62 (0.03, 37.01)[c]	>0.99[c]		
GG	0.98	0.97	*Reference*			
GT	0.02	0.03	1.78 (0.18, 17.45)	0.62	0.59 (0.05, 7.07)	0.68
TT	Nil	Nil	NA	NA	NA	NA
GT+TT	0.02	0.03	1.78 (0.18, 17.45)	0.62	0.59 (0.05, 7.07)	0.68
INDIAN						
T allele	0.31	0.20	1.82 (0.84, 3.95)[c]	0.18[c]		
GG	0.47	0.65	*Reference*			
GT	0.45	0.31	**2.39 (1.03, 5.54)**	**0.04**	**2.54 (1.07, 6.04)**	**0.03**
TT	0.08	0.04	1.18 (0.30, 4.64)	0.82	1.62 (0.40, 6.65)	0.5
GT+TT	0.54	0.35	2.09 (0.97, 4.50)	0.06	**2.32 (1.05, 5.14)**	**0.04**

[a] Obtained using logistic regression analysis, unless otherwise stated; [b] Adjusted for age, sex and BMI; [c] Obtained using Chi-square test

(iii) rs11196205

rs11196205 C>G	T2DM	Non-T2DM	OR (95% CI)[a]	p-value	Adjusted OR (95% CI)[b]	Adjusted p-value
OVERALL						
G allele	0.16	0.11	**1.62 (1.22, 2.13)**	**0.0009**[c]		
CC	0.73	0.81	*Reference*			
CG	0.22	0.16	**1.60 (1.14, 2.25)**	**0.006**	1.41 (0.98, 2.01)	0.06
GG	0.05	0.03	1.99 (0.96, 4.11)	0.06	1.76 (0.80,3.87)	0.09
CG+GG	0.27	0.19	**1.66 (1.21, 2.27)**	**0.002**	**1.46 (1.03, 2.04)**	**0.03**
MALAY						
G allele	0.11	0.10	1.11 (0.75, 1.62)	0.68		
CC	0.80	0.82	*Reference*			
CG	0.18	0.16	1.30 (0.85, 1.98)	0.23	1.24 (0.80, 1.92)	0.34
GG	0.02	0.02	0.99 (0.31, 3.18)	0.99	1.08 (0.31, 3.81)	0.91
CG+GG	0.20	0.18	1.26 (0.84, 1.89)	0.26	1.22 (0.80, 1.87)	0.35
CHINESE						
G allele	0.01	0.02	0.93 (0.07, 49.64)	>0.99		
CC	0.97	0.97	*Reference*			
CG	0.03	0.03	0.58 (0.14, 2.38)	0.45	0.72 (0.07, 7.80)	0.79
GG	Nil	Nil	NA	NA	NA	NA
CG+GG	0.03	0.03	0.72 (0.19, 2.78)	0.63	0.72 (0.07, 7.80)	0.79
INDIAN						
G allele	0.41	0.33	1.45 (0.74, 2.82)	0.35		
CC	0.34	0.48	*Reference*			
CG	0.50	0.39	1.69 (0.76, 3.74)	0.2	1.70 (0.75, 3.87)	0.21
GG	0.16	0.13	1.60 (0.52, 4.86)	0.41	1.72 (0.54, 5.47)	0.36
CG+GG	0.67	0.52	1.66 (0.79, 3.49)	0.18	1.71 (0.79, 3.67)	0.17

[a] Obtained using logistic regression analysis, unless otherwise stated; [b] Adjusted for age, sex and BMI; [c] Obtained using Chi-square test

(iv) rs7901695

rs7901695 T>C	T2DM	Non-T2DM	OR (95% CI)[a]	p-value	Adjusted OR (95% CI)[b]	Adjusted p-value
OVERALL						
C allele	0.11	0.07	**1.71 (1.17, 2.50)**	**0.007**[c]		
TT	0.79	0.87	*Reference*			
TC	0.19	0.12	**1.95 (1.31, 2.90)**	**0.001**	**1.73 (1.14, 2.63)**	**0.01**
CC	0.02	0.01	1.91 (0.69, 5.31)	0.21	1.66 (0.57, 4.80)	0.35
TC+CC	0.21	0.13	**1.94 (1.33, 2.83)**	**0.001**	**1.72 (1.16, 2.56)**	**0.007**
MALAY						
C allele	0.07	0.06	1.21 (0.76, 1.93)	0.50		
TT	0.86	0.88	*Reference*			
TC	0.14	0.11	1.44 (0.89, 2.36)	0.14	1.44 (0.86, 2.40)	0.17
CC	<0.01	0.01	0.71 (0.10, 5.07)	0.73	0.78 (0.11, 5.63)	0.8
TC+CC	0.14	0.12	1.39 (0.86, 2.25)	0.17	1.39 (0.84, 2.29)	0.2
CHINESE						
C allele	0.02	0.10	**0.19 (0.04, 0.92)**	**0.04**		
TT	0.96	0.79	*Reference*			
TC	0.04	0.21	0.54 (0.15, 1.96)	0.35	**0.19 (0.04, 0.80)**	**0.02**
CC	Nil	Nil	NA	NA	NA	NA
TC+CC	0.04	0.21	0.54 (0.15, 1.96)	0.35	**0.19 (0.04, 0.80)**	**0.02**
INDIAN						
C allele	0.34	0.14	**3.15 (1.05, 9.43)**	**0.05**		
TT	0.42	0.79	*Reference*			
TC	0.48	0.14	**3.80 (1.32, 10.93)**	**0.01**	**4.80 (1.59, 14.44)**	**0.005**
CC	0.10	0.07	1.55 (0.40, 6.06)	0.53	1.96 (0.46, 8.34)	0.36
TC+CC	0.58	0.21	**2.96 (1.20, 7.29)**	**0.02**	**3.77 (1.45, 9.79)**	**0.007**

[a] Obtained using logistic regression analysis, unless otherwise stated; [b] Adjusted for age, sex and BMI; [c] Obtained using Chi-square test

(v) rs4506565

rs4506565 A>T	T2DM	Non-T2DM	OR (95% CI)[a]	p-value	Adjusted OR (95% CI)[b]	Adjusted p-value
OVERALL						
T allele	0.13	0.07	**1.97 (1.35, 2.88)**	**0.0005**[c]		
AA	0.78	0.88	*Reference*			
AT	0.19	0.11	**2.10 (1.42, 3.12)**	**2.1e-4**	**1.92 (1.26, 2.91)**	**0.002**
TT	0.03	0.01	2.49 (0.93, 6.70)	0.07	2.19 (0.79, 6.05)	0.13
AT+TT	0.22	0.12	**2.15 (1.48, 3.12)**	**0.001**	**1.95 (1.32, 2.90)**	**0.001**
MALAY						
T allele	0.07	0.06	1.21 (0.76, 1.93)	0.50		
AA	0.86	0.88	*Reference*			
AT	0.14	0.11	1.44 (0.89, 2.36)	0.14	1.44 (0.86, 2.40)	0.17
TT	<0.01	0.01	0.71 (0.10, 5.07)	0.73	0.78 (0.11, 5.63)	0.8
AT+TT	0.14	0.12	1.39 (0.86, 2.25)	0.17	1.39 (0.84, 2.29)	0.2
CHINESE						
T allele	0.02	0.08	0.28 (0.06, 1.45)	0.14		
AA	0.96	0.83	*Reference*			
AT	0.03	0.17	0.39 (0.10, 1.52)	0.18	**0.17 (0.03, 0.87)**	**0.03**
TT	0.01	Nil	NA	NA	NA	NA
AT+TT	0.04	0.17	0.49 (0.14, 1.77)	0.28	**0.22 (0.05, 1.0)**	**0.05**
INDIAN						
T allele	0.37	0.13	**3.83 (1.30, 11.33)**	**0.02**		
AA	0.38	0.80	*Reference*			
AT	0.50	0.13	**4.71 (1.66, 13.37)**	**0.004**	**5.80 (1.97, 17.11)**	**0.001**
TT	0.12	0.07	2.02 (0.54, 7.65)	0.3	2.57 (0.64, 10.35)	0.18
AT+TT	0.62	0.20	**3.71 (1.53, 9.0)**	**0.004**	**4.61 (1.82, 11.72)**	**0.001**

[a] Obtained using logistic regression analysis, unless otherwise stated; [b] Adjusted for age, sex and BMI; [c] Obtained using Chi-square test

3.3. INTERACTION BETWEEN SNPS AND ASSOCIATION WITH T2DM

3.3.1. Linkage analysis

Linkage analysis analyzes the inter-allelic relationship of different SNPs on the *TCF7L2* gene, especially in terms of heritability. This is established by using the LOD score (logarithm [base 10] of odds). Table 3.4(i) tabulates the minor allele of the SNPs against each other, with the LOD score displayed for the degree of linkage. It is of note that any LOD of more than +3.0 is considered to denote possibility of linkage.

The highest LOD was observed between alleles C of rs7901695and C of rs11196205 (306.88), denotes high probability of linkage) whereas the lowest score was between alleles C or rs11196205 and T of rs12255372 (6.86, denotes low probability of linkage).

3.3.2. Linkage disequilibrium

Linkage disequilibrium (LD) is the non-random association of alleles at more than one locus on different parts of the chromosome or DNA. This is measured using two scores: D' and r^2. Table 3.4(ii) shows these scores for the observed SNPs in *TCF7L2*. The highest LD was observed between alleles T of rs4506565 and T of rs7903146 (highest D' and r^2), whereas the scores between the T allele of rs7903146 and C allele of rs11196205 showed the weakest LD (lowest D' and r^2).

The LD plot produced in Haploview is illustrated in Figure 3.2, showing increasing strength of LD with numbers closer to 100. According to this figure, relationship between SNPs rs4506565 and rs7903146 produced the highest LD, followed by the relationships between rs4506565 and rs12255372, rs7903146 and rs12255372, and finally rs7901695 and rs11196205.

Table 3.4: Interaction between SNPs in *TCF7L2*

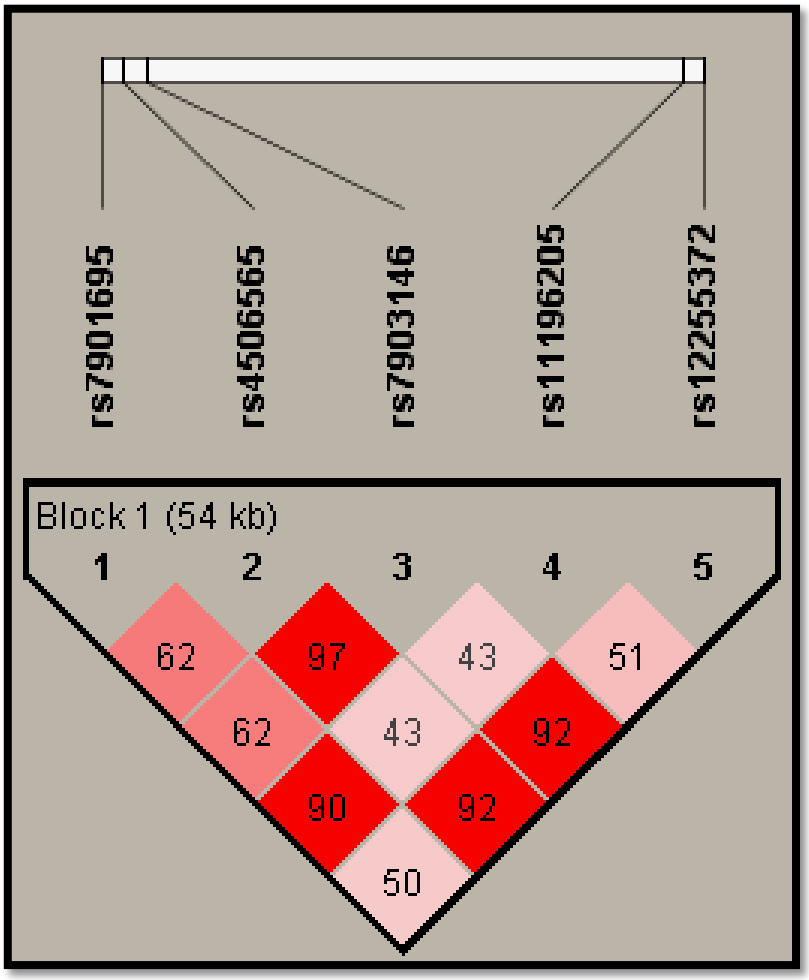
(i) LOD scores

SNP: Minor Allele	**rs7901695: C**	**rs4506565: T**	**rs7903146: T**	**rs11196205: C**	**rs12255372: T**
rs7901695: C		14.46	13.97	306.88	7.07
rs4506565: T	14.46		206.27	7.05	123.76
rs7903146: T	13.97	206.27		7.03	124.73
rs11196205: C	306.88	7.05	7.03		6.86
rs12255372: T	7.07	123.76	124.73	6.86	

(ii) D’ and r2 scores

	SNP: Minor Allele	**D’**				
		rs7901695: C	**rs4506565: T**	**rs7903146: T**	**rs11196205: C**	**rs12255372: T**
r^2	**rs7901695: C**		0.625	0.621	0.901	0.5
	rs4506565: T	0.096		0.977	0.436	0.925
	rs7903146: T	0.094	0.949		0.435	0.926
	rs11196205: C	0.752	0.042	0.041		0.519
	rs12255372: T	0.047	0.655	0.66	0.045	

Figure 3.2: LD plot showing the D' scores of SNPs in *TCF7L2*



SNP1, rs7901695; SNP2, rs4506565; SNP3, rs7903146; SNP4, rs11196205; SNP5, rs12255372; kb, kilobasepairs.

3.4.3. Haplotype analysis

According to Table 3.5, there are several possible haplotype blocks. These were chosen using Figure 3.2 as reference, where the SNPs exhibiting high LD were included in the construction of the haplotype blocks. All haplotypes with overall frequencies exceeding 5% were analysed for difference in distribution.

The first block consisted of rs4506565 and rs7903146, which achieved a D' of 97, and hence is in strong LD. Two possible haplotypes were AC and TT. For haplotype AC, there were a lower proportion of the cases carrying this haplotype combination compared to the controls (p=4.0e-4). Carriers of this haplotype were 1.82 times more likely to be non-diabetic. In contrast, carriers of both minor alleles for the SNPs, the TT haplotype, were more prevalent in the cases compared to controls (p=9.97e-5), with the carriers 1.98 times more likely to have T2DM.

The second block consisted of rs7903146 and rs12255372 (D' 92), with rs11196205 also included in the analysis as it was located in between the other 2 SNPs. There were 3 haplotypes that was prevalent in this block. The first haplotype, CGG, was less prevalent in the cases compared to controls (p=1.35e-5), which carried 1.54 times less risk of having T2DM for its carriers. The next two haplotypes were associated with an increased risk of developing T2DM. The CCG haplotype is slightly more prevalent in the cases compared to controls (p=0.05), with an increased risk of 1.23 of having T2DM. The TCT haplotype contained two minor alleles, was more prevalent in the cases compared to controls (p=0.03) and carried 1.71 times more risk of having T2DM.

Block 3 contained 4 SNPs, which are rs7901695, rs4506565, rs7903146 and rs11196205. Strong LD was observed between rs7901695 and rs11196205 (D' 90). As for the other SNPs, they were in strong LD with each other, but not with the other two. The first haplotype TACG, which contained all the major alleles except for one, was associated

with 1.54 times less risk of having T2DM (p=1.76e-5). The other two prevalent haplotypes were associated with an increased risk of T2DM. CACC contained only one minor allele, but increased the risk of T2DM by 1.33 times in its carriers (p=0.015). The final haplotype in this block, CTTG, contained 3 minor alleles. It was more prevalent in the cases compared to controls, with its carriers 1.66 times more likely to have T2DM (p=0.018).

In the 4th block, there were rs4506565, rs7903146, rs11196205 and rs12255372. Besides having strong LD between each other, rs4506565 and rs7903146 also formed strong LDs with rs12255372 (each D' is 90). The most prevalent haplotype, ACGG, appears to have a protective effect (p=1.63e-5). Carriers of this haplotype have 1.54 times less risk of having T2DM. Carrying the ACCG haplotype lead to 1.25 times higher risk of having T2DM (p=0.04), whereas carriers of the TTCT haplotype were 1.68 times more likely to have T2DM (p=0.02).

Table 3.5: Haplotype analysis of LD blocks in *TCF7L2* gene

Haplotype block	Size	Possible Haplotypes	Frequency Overall	Frequency Case	Frequency Control	OR (95% CI) [a]	p-value [a]
1: (rs4506565 & rs790316)	2kbp	AC	0.9	*0.880*	*0.930*	*0.55 (0.39, 0.76)*	*4.0e-4*
		TT	0.1	**0.117**	**0.063**	**1.98 (1.41, 2.84)**	**9.97e-5**
2: (rs7903146, rs11196205 & rs12255372	50kbp	CGG	0.63	*0.593*	*0.692*	*0.65 (0.53, 0.79)*	*1.35e-5*
		CCG	0.27	**0.281**	**0.241**	**1.23 (1.0, 1.52)**	**0.05**
		TCT	0.05	**0.057**	**0.034**	**1.71 (1.06, 4.26)**	**0.03**
3: (rs7901695, rs4506565, rs7903146, & rs11196205)	52kbp	TACG	0.62	*0.583*	*0.682*	*0.65 (0.54, 0.79)*	*1.76e-5*
		CACC	0.23	**0.251**	**0.202**	**1.33 (1.06, 1.66)**	**0.015**
		CTTC	0.06	**0.071**	**0.045**	**1.66 (1.09, 2.52)**	**0.018**
4: (rs4506565, rs7903146, rs11196205 & rs12255372)	52kbp	ACGG	0.63	*0.592*	*0.691*	*0.65 (0.54, 0.79)*	*1.63e-5*
		ACCG	0.27	**0.280**	**0.238**	**1.25 (1.01, 1.54)**	**0.04**
		TTCT	0.05	**0.056**	**0.034**	**1.68 (1.05, 2.70)**	**0.02**

[a] Analysed for significance using χ^2 test; letters in red are minor alleles

Bold: significant causative association; *Italics*: significant protective association

3.4. SNPS AND T2DM TREATMENT

3.4.1. Treatment regimes

From Table 3.6 below, it was observed that the majority of patients were on some form of oral antidiabetic agent only therapy. The combination of antidiabetics and insulin was the second most common treatment regime, followed by insulin only regime. Among patients on oral antidiabetic agents only, usage of the combination of metformin and a sulfonylurea ranked highest, followed by monotherapies using metformin or sulfonylureas only.

Table 3.6: Treatment regimes used in T2DM patients in the study population

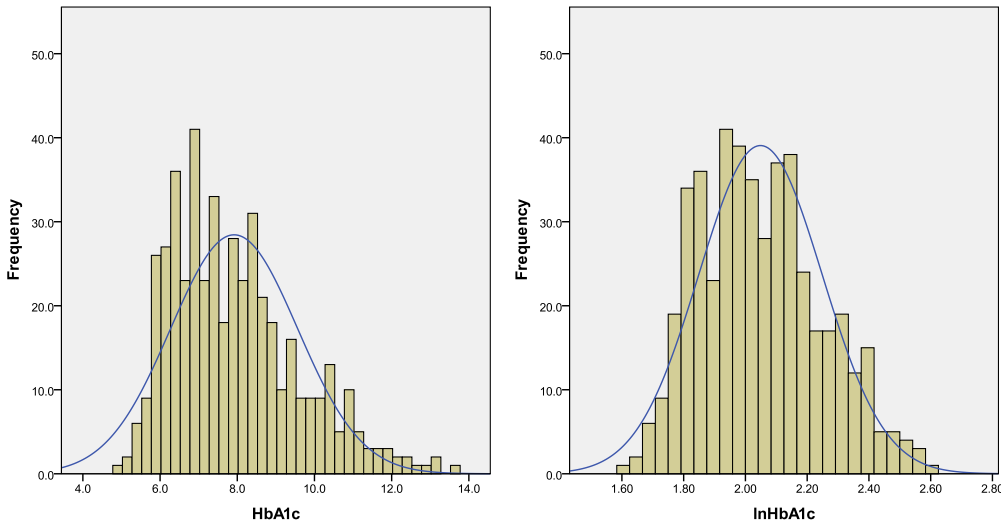
Treatment regime	**n**	**Percentage**
Diet control only	8	2
Antidiabetic agents only	329	66.7
Metformin only	*81*	*24.6*
Sulfonylurea only	*38*	*11.6*
Metformin and sulfonylurea	*181*	*55.0*
Other combinations of oral agents	*29*	*8.8*
Insulin only	48	9.7
Insulin and oral agent combination	106	21.6

3.4.2. Glycated haemoglobin fraction (HbA_{1c})

The distribution and measures of centrality and peakedness of the values of HbA_{1c} is illustrated in Figure 3.22 below. In the unchanged data (HbA_{1c}), there was significant skewness and kurtosis observed. However after transformation (natural log transformation, $Ln(HbA_{1c})$, these measures indicated normal distribution as indicated by the histogram, and thus parametric testing was used to test for significant differences between groups.

Figure 3.3: Distribution of HbA_{1c} values in T2DM subjects

Variable	Skewness	SE of Skewness	Kurtosis	SE of Kurtosis	Mean	Median
HbA_{1c}	0.809	0.113	0.283	0.225	7.918	7.700
$ln(HbA_{1c})$	0.370	0.113	-0.456	0.225	2.049	2.041



3.4.3. Characteristics according to treatment regimes

According to Table 3.6, there were 4 main treatment regimes. As depicted in Table 3.7, the proportion of male subjects in the diet only and insulin only groups were slightly higher than female (p=0.12). There were no significant differences in ethnic group distribution between the treatment regimes. There were also no significant difference between mean age and proportion of subjects aged more than 55 years between the treatment regimes.

The mean BMI was highest in the insulin only treatment group, whilst the lowest was observed in the diet only group (p=0.001). Similarly, mean BF% was also highest in the insulin only group and lowest in subjects on diet only (p=0.01). This pattern was reflected in the proportion of subjects with a BMI of more than 23kgm-2 (p=0.02) but not in the proportion of subjects with BF% in the obese range (>25% in males, >33% in females; p=0.68).

The lowest mean HbA_{1c} was observed in the diet only treatment group. Among the pharmacological treatment groups, the lowest mean HbA_{1c} was observed in the oral antidiabetic agent only regime, followed by insulin only and finally combination regime (p<0.0001). The highest proportion of subjects achieving HbA_{1c} among the pharmacological treatment groups was found in the oral antidiabetic agents only treatment regime, whilst the lowest was found in the combination treatment regime (p=4.5e-8).

Table 3.7: Comparisons between the subjects in the various treatment regime of T2DM

Variable	Treatment Regime				p-value[a]
	Diet only	Antidiabetic agent only	Insulin only	Antidiabetic agent and insulin combination	
Male proportion (%)	66.7	49.5	58.3	40.6	0.12
Ethnic group distribution (Malay/Chinese/Indian) (%)	40/40/20	60.5/17.9/21.6	60.4/12.5/27.1	68.9/18.9/12.3	0.12
Mean age (years±SD)	56.9±9.6	57.1±7.9	57.7±9.0	55.6±8.1	0.34
Proportion >55 years (%)	66.7	64.4	64.6	64.2	0.9
Mean BMI (kgm^{-2}±SD)	24.0±2.3	27.3±5.7	30.5±6.4	28.1±5.6	**0.001**
Overweight* proportion (%)	66.7	78.1	95.7	84.0	**0.02**
Mean BF% (%±SD)	29.3±6.0	35.1±8.5	38.2±9.7	36.7±9.0	**0.01**
Proportion obese** (%)	88.9	85.4	95.7	84.9	0.68
Mean HbA_{1c}	6.3±0.8	7.5±1.5	8.5±1.9	8.8±1.5	**6.4e-16**[b]
Proportion achieve HbA_{1c} target*** (%)	75.0	28.6	18.6	4.9	**4.5e-8**

*BMI>23kgm^{-2}; **BF%>25% (male) or >33% (female); *** HbA_{1c}<6.5%; [a] Data tested for significance using ANOVA (continuous), χ^2 (categorical) or Spearman correlational tests (where appropriate); [b] Obtained after natural log (ln) transformation; significant values in **bold**
BMI, body mass index; BF%, body fat percentage; HbA_{1c}, glycated haemoglobin fraction

3.4.4. HbA_{1c} levels according to TCF7L2 genotype

All patients receiving insulin, either alone or as part of a treatment regime, was grouped together in an effort to increase subject number for a meaningful analysis of HbA_{1c} across genotypes of the SNPs. Together with subjects taking oral antidiabetic agents only, this analysis is presented in Table 3.8. Generally, presence of a variant allele leads to a higher mean HbA_{1c} in most of the SNPs examined, regardless of treatment regime. A significantly higher HbA_{1c} was observed in carriers of TT genotype in rs4506565 compared to their AA counterparts (p=0.03). All other observations were statistically insignificant.

Due to the higher number of subjects on treatment with oral antidiabetic agents, this treatment regime was chosen to be analysed further. Figure 3.4 shows the differences in HbA_{1c} levels according to genotype. As per Table 3.6, the most number of subjects in this treatment regime was on treatment with a combination of metformin and a sulfonylurea type drug.

In the first SNP examined (rs7903146), it was noted that as a general rule, the HbA_{1c} values of subjects carrying the variant genotypes were higher than their wildtype counterparts. In particular, those carrying the CT genotype in the metformin-sulfonylurea combination had a significantly higher HbA_{1c} value than the CC carriers, even after adjusting for age and BMI (p=0.007). There was an absence of carriers of the TT genotypes in all other treatment group except the metformin-sulfonylurea combination group.

For rs12255372, it was noted that variant allele carriers had lower HbA_{1c} values compared to the wildtype in all treatment groups except for subjects treated with the metformin-sulfonylurea combination. However, these observations did not reach statistical significance. In contrast, TT carriers in the metformin-sulfonylurea combination group

had a significantly higher HbA_{1c} value compared to GG carriers (p=0.03). However, the significance diminished after correcting for age and BMI.

Variant allele carriers of rs11196205 in all but the metformin-sulfonylurea combination treatment group had lower HbA_{1c} values than their wildtype counterparts. However, the differences did not achieve statistical significance. In the combination group, HbA_{1c} levels of both CG and GG genotype carriers were higher than the CC carriers, with the difference being statistically significant for the former (adjusted p=0.003).

For rs7901695, variant allele carriers also had higher HbA_{1c} levels compared to their wildtype counterparts. Apart from subjects treated with the metformin-sulfonylurea combination, the differences observed were not significant. In the combination group, the difference of HbA_{1c} between the TC and TT genotype achieved statistical significance, even after adjustment for age and BMI was made (p=0.002).

Finally in rs4506565, a similar observation to rs7901695 was noted. In the metformin-sulfonylurea combination group, AT carriers had significantly higher HbA_{1c} levels compared to AA (p=0.003). The significance of this difference did not diminish after correcting for subjects' age and BMI. In both these SNPs (rs7901695 and rs4506565), heterozygous carriers in the other oral antidiabetic combinations group had lower HbA_{1c} compared to their wildtype genotypes, albeit insignificant.

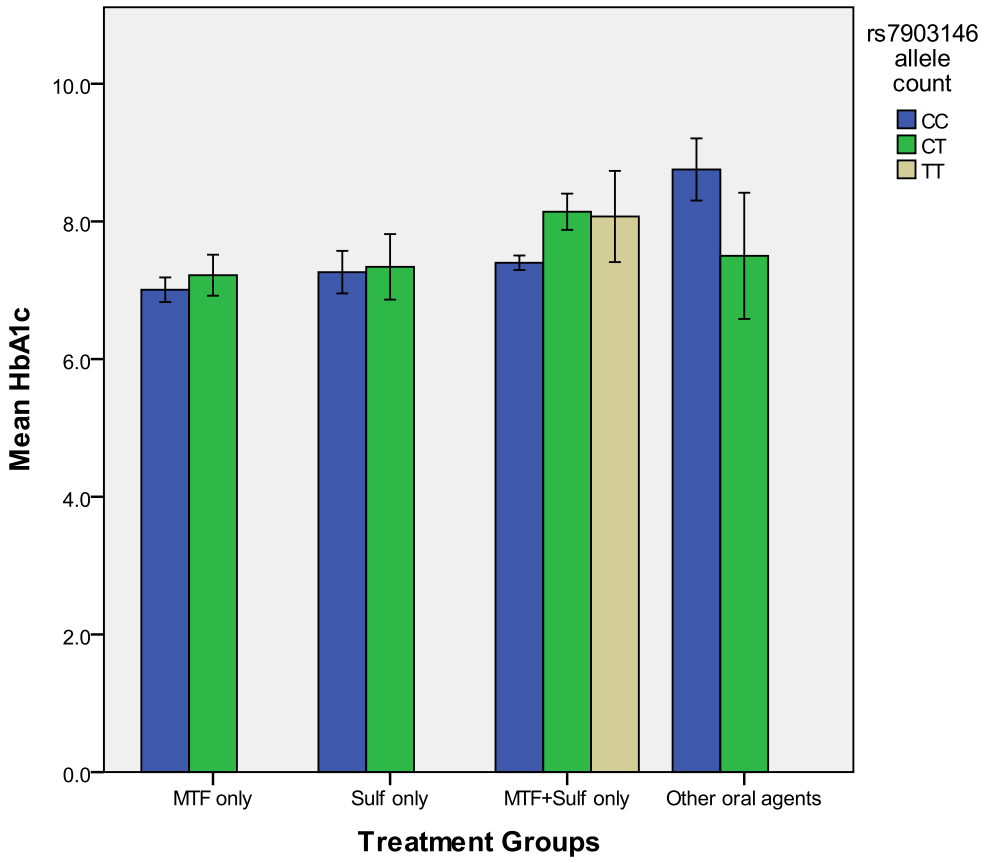
Table 3.8: Comparison of HbA_{1c} according to *TCF7L2* SNP genotypes in two treatment regimes

SNP/ Genotype		HbA_{1c} (%, mean±SD) in each treatment regime	
		Oral antidiabetic agent only	**All insulin therapy**
	n	329	154
rs7903146	CC*	7.42±1.45	8.65±1.59
	CT	7.77±1.41	8.83±1.47
	CT	8.07±1.75	11.03±1.44
	p-value[a]	*CT=0.31, TT=0.73*	*CT=1.0, TT=0.06*
rs12255372	GG*	7.49±1.45	8.63±1.58
	GT	7.46±1.43	9.06±1.58
	TT	8.17±1.64	10.67±1.36
	p-value[a]	*GT=1.0, TT=0.67*	*GT=0.77, TT=0.12*
rs11196205	CC*	7.45±1.45	8.63±1.60
	CG	7.70±1.53	8.97±1.52
	GG	7.65±1.49	9.52±1.95
	p-value[a]	*CG=0.71, GG=1.0*	*CG=0.87, GG=0.76*
rs7901695	TT*	7.44±1.47	8.64±1.59
	TC	7.88±1.43	8.85±1.49
	CC	8.07±1.75	11.03±1.44
	p-value[a]	*TC=0.11, CC=0.76*	*TC=1.0, CC=0.06*
rs4506565	AA*	7.42±1.45	8.65±1.58
	AT	7.83±1.42	8.76±1.52
	TT	7.64±1.54	**10.9±1.20**
	p-value[a]	*AT=0.13, TT=1.0*	*AT=1.0,* ***TT=0.03***

* Reference genotype, [a]after natural log (ln) transformation, **bold** indicates significant observation

Figure 3.4: Comparisons of HbA_{1c} levels according to genotype in each oral antidiabetic treatment group

(i) rs7903146

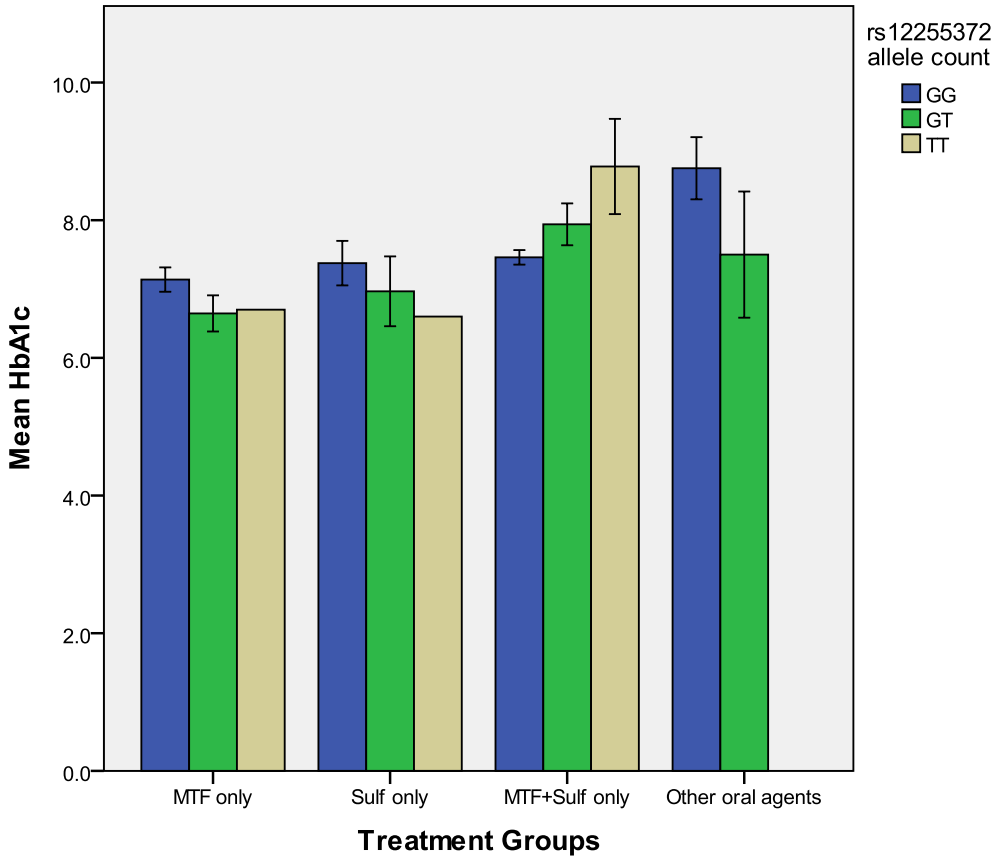


Oral antidiabetic regime	rs7903146 genotype			p-value[a] CT/TT	Adjusted p-value[b] CT/TT
	CC	*CT*	*TT*		
Metformin only	7.00±1.33	7.22±1.19	nil	0.48	0.5
Sulfonylurea only	7.26±1.61	7.34±1.06	nil	0.81	0.42
Metformin-Sulfonylurea combination	7.40±1.17	8.14±1.49	8.07±1.75	**0.005**/0.21	**0.007**/0.34
Other combinations	8.76±2.12	7.50±1.59	nil	0.32	0.63

[a] Univariate analysis on ln(HbA_{1c}) with CC as reference category; [b] Univariate analysis adjusted for age and BMI

Data expressed as mean±SEM; significant values are **bold**

(ii) rs12255372

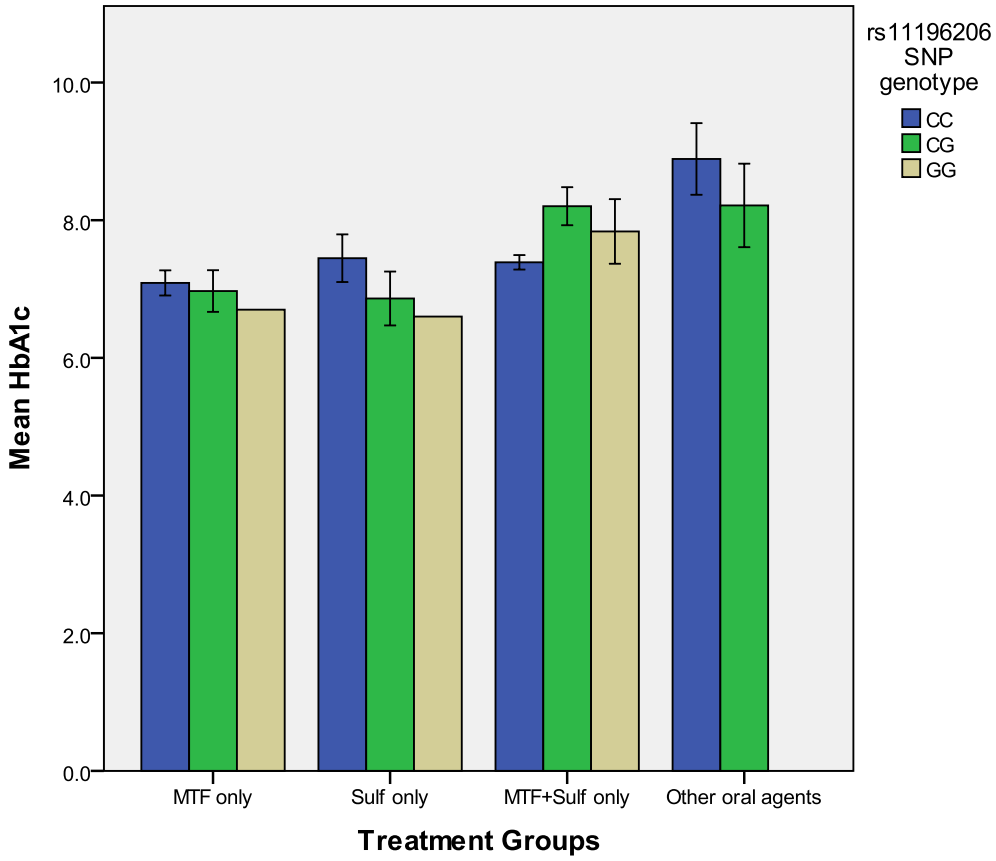


Oral antidiabetic regime	rs12255372 genotype			p-value[a] GT/TT	Adjusted p-value[b] GT/TT
	GG	*GT*	*TT*		
Metformin only	7.14±1.36	6.65±0.87	6.70	0.25/0.78	0.25/0.78
Sulfonylurea only	7.38±1.62	6.97±1.24	6.60	0.58/0.65	0.93/0.8
Metformin-Sulfonylurea combination	7.46±1.21	7.94±1.52	8.78±1.55	0.10/**0.03**	0.1/0.08
Other combinations	8.76±2.12	7.5±1.59	nil	0.32	0.63

[a] Univariate analysis on ln(HbA_{1c}) with GG as reference category; [b] Univariate analysis adjusted for age and BMI

Data expressed as mean±SEM; significant values are **bold**

(iii) rs11196205

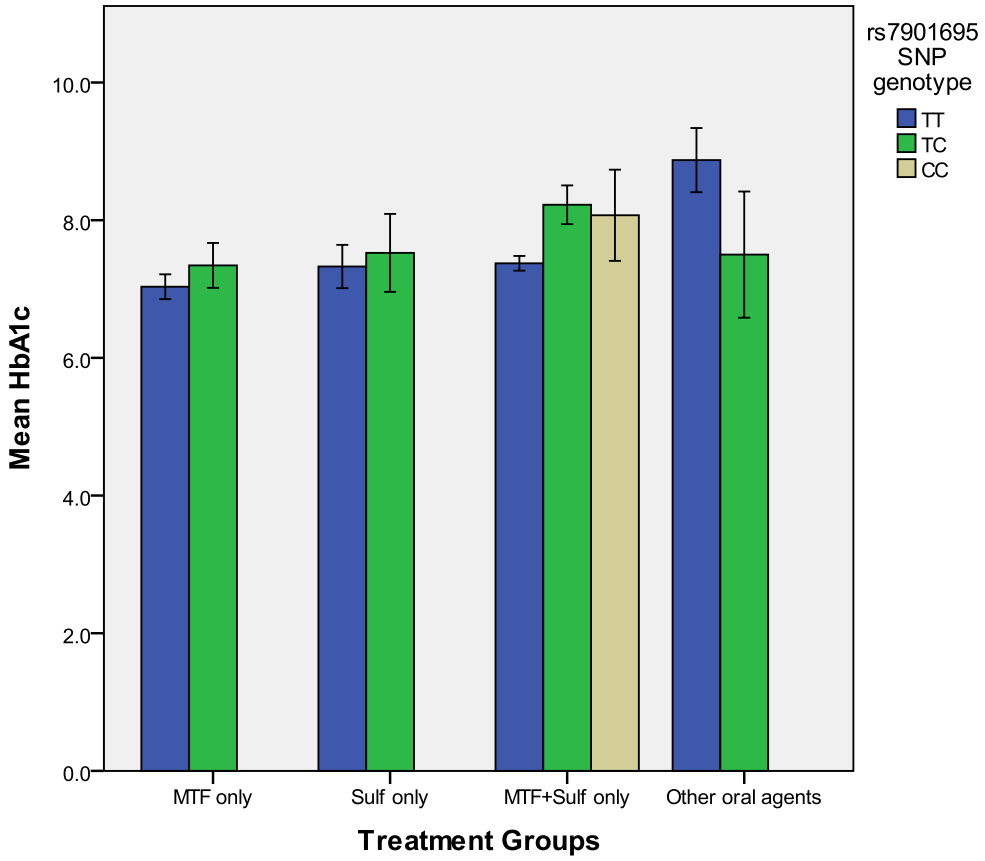


Oral antidiabetic regime	rs11196205 genotype			p-value[a] CG/GG	Adjusted p-value[b] CG/GG
	CC	*CG*	*GG*		
Metformin only	7.09±1.33	6.97±1.25	6.70	0.75/0.81	0.81/0.81
Sulfonylurea only	7.45±1.66	6.86±1.11	6.60	0.37/0.61	0.65/0.75
Metformin-Sulfonylurea combination	7.39±1.15	8.20±1.54	7.84±1.56	**0.003**/0.3	**0.003**/0.57
Other combinations	8.89±2.27	8.21±1.60	nil	0.54	0.48

[a] Univariate analysis on ln(HbA_{1c}) with CC as reference category; [b] Univariate analysis adjusted for age and BMI

Data expressed as mean±SEM; significant values are **bold**

(iv) rs7901695

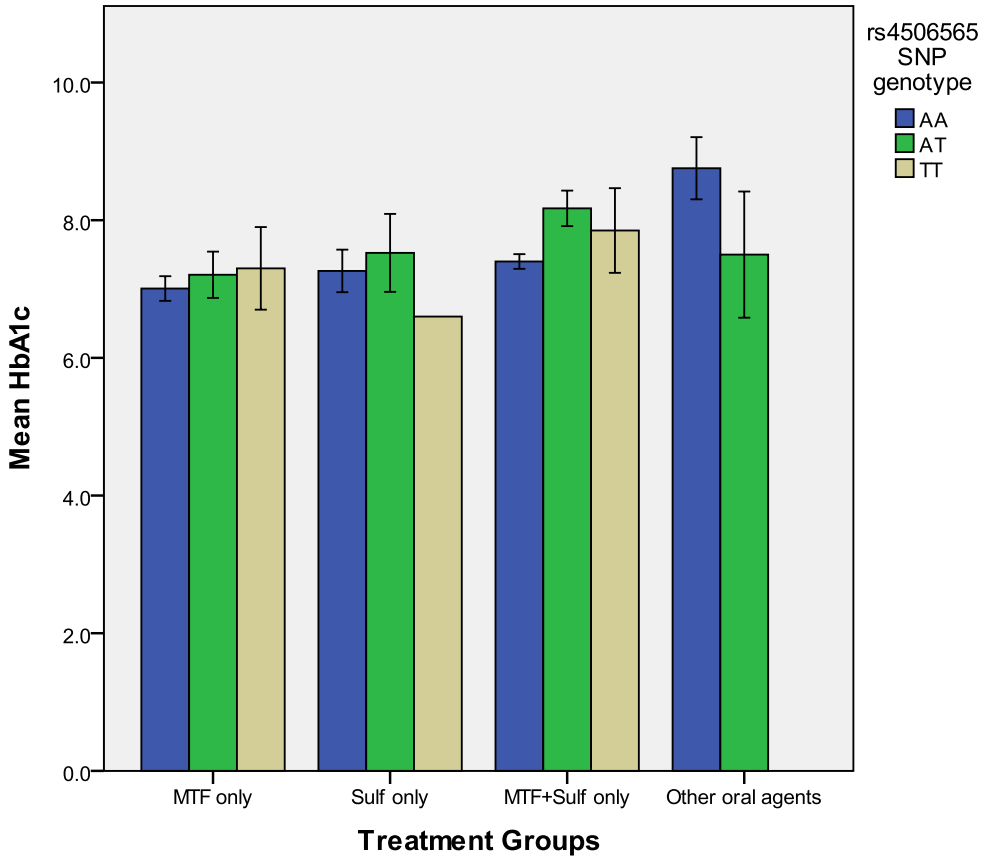


Oral antidiabetic regime	rs7901695 genotype			p-value[a] TC/CC	Adjusted p-value[b] TC/CC
	TT	*TC*	*CC*		
Metformin only	7.03±1.33	7.34±1.22	nil	0.36	0.37
Sulfonylurea only	7.33±1.60	7.53±1.13	nil	0.71	0.4
Metformin-Sulfonylurea combination	7.37±1.16	8.22±1.51	8.07±1.75	**0.002**/0.19	**0.002**/0.32
Other combinations	8.87±2.19	7.50±1.59	nil	0.3	0.74

[a] Univariate analysis on ln(HbA_{1c}) with TT as reference category; [b] Univariate analysis adjusted for age and BMI

Data expressed as mean±SEM; significant values are **bold**

(v) rs4506565



Oral antidiabetic regime	rs4506565 genotype			p-value[a] AT/TT	Adjusted p-value[b] AT/TT
	AA	*AT*	*TT*		
Metformin only	7.01±1.33	7.21±1.26	7.30±0.85	0.54/0.66	0.56/0.67
Sulfonylurea only	7.26±1.61	7.53±1.13	6.60	0.65/0.71	0.32/0.88
Metformin-Sulfonylurea combination	7.40±1.17	8.17±1.46	7.85±1.74	**0.003**/0.39	**0.003**/0.63
Other combinations	8.76±2.12	7.50±1.59	nil	0.32	0.63

[a] Univariate analysis on ln(HbA_{1c}) with AA as reference category; [b] Univariate analysis adjusted for age and BMI

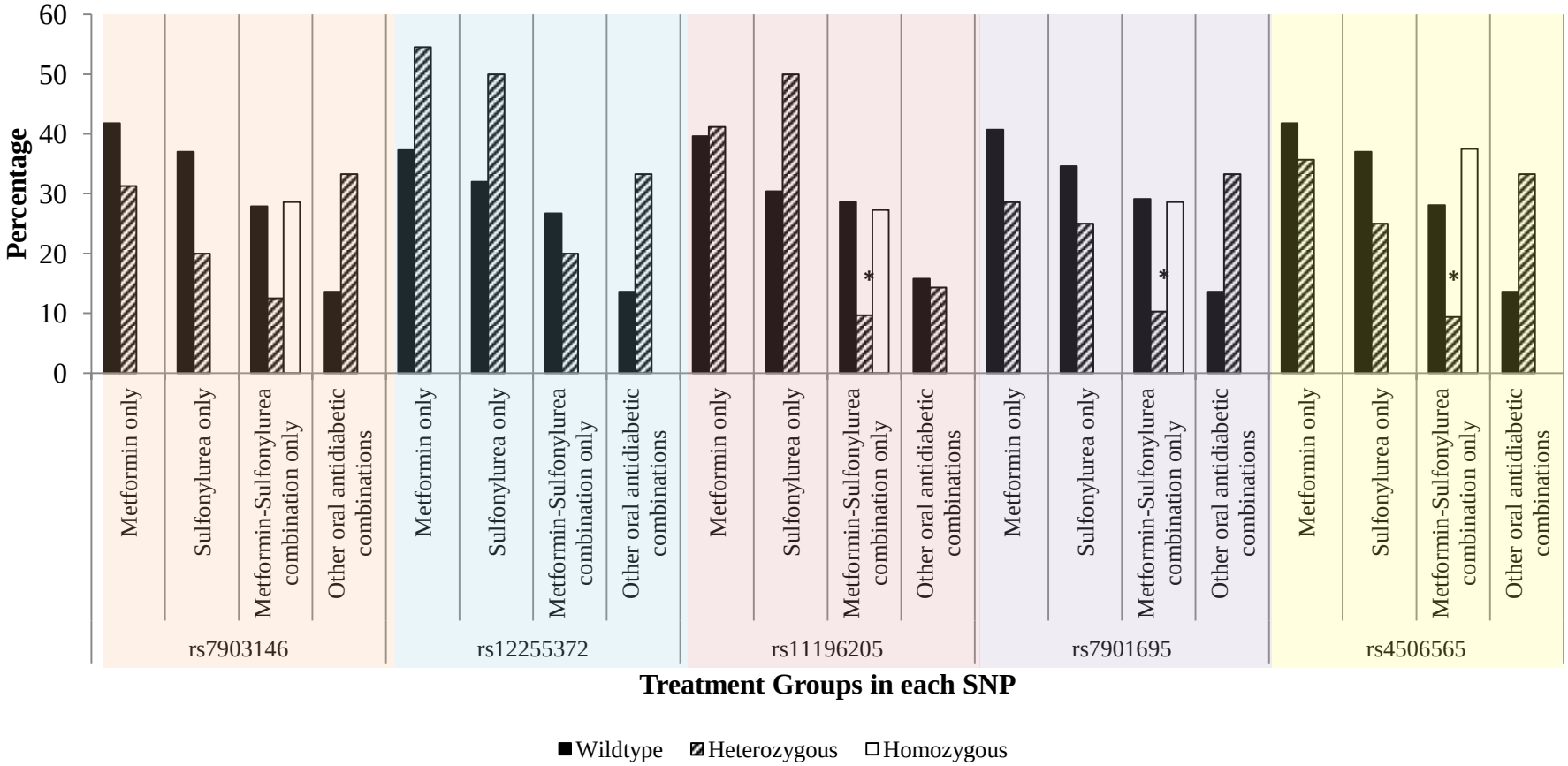
Data expressed as mean±SEM; significant values are **bold**

3.4.5. Achievement of HbA_{1c} target in oral antidiabetic treatment regime

Figure 3.5 below illustrates the differences in proportion of subjects achieving the HbA_{1c} target of less than 6.5% in each oral antidiabetic treatment group according to their genotypes.

Significant differences were observed in rs11196205, rs7901695 and rs4506565 for the respective metformin-sulfonylurea treatment group ($p<0.05$ for all). In these SNPs, there were a significantly smaller proportion of heterozygous carriers achieving the target compared to their wildtype counterparts. For rs11196205, GC carriers were 3.86 times less likely to achieve HbA_{1c} target compared to CC genotype (95% CI 1.05 – 14.29, p=0.04). TC carriers of rs7901695 were 3.98 times less likely to achieve the target compared to their TT carriers (95% CI 1.04 – 15.38, p=0.04). Finally, AT carriers of rs4506565 were found to be 4.18 times less likely to achieve HbA_{1c} target compared to the AA carriers (95% CI 1.10 – 15.87, p=0.035).

Figure 3.5: Percentage of subjects achieving HbA_{1c} <6.5% in each treatment group, according to genotype



* p<0.05 vs wildtype, after logistic regression adjusted for age and BMI; each bar represents the percentage of patients carrying the genotype that achieves HbA_{1c} of 6.5% or less

CHAPTER 4

DISCUSSION

4.1. SNPS IN *TCF7L2* AND BASIC CHARACTERISTICS OF STUDY SUBJECTS

The most obvious and significant difference observed among cases and controls is the difference in mean age. Overall, cases were 3.6 years older than controls. The biggest difference in mean age was observed in the Chinese subjects (6.3 years, p=0.01), whilst the difference in the Malays were the smallest observed (2.9 years, p<0.0001). In each cases and controls, there were no significant differences in mean age when compared according to genotypes. Similarly, there was no significant difference in the proportion of the subjects aged more than 55 years when compared according to genotypes in both groups.

The difference in mean age between the cases and controls reflects the difficulty in getting non-T2DM subjects during the recruitment stage. Initially, healthy subjects were obtained from participants of UMMC's blood donation drives around Klang Valley. This lead to the accumulation of control subjects who were mostly young professionals. By incorporating subjects from UMMC who were non-T2DM as well, the mean age difference between the two groups was reduced considerably.

Physiologically, pancreatic beta cell function deteriorates with advancing age (A. M. Chang & Halter, 2003). This is attributed to the increased incidence and prevalence of T2DM in the elderly compared to the young, irrespective of ethnicity or geographical location (Q. Qiao et al., 2003). Furthermore, the increased abdominal adiposity observed with advancing age also contributed towards the increase in prevalence of T2DM according to age (Lutsey, Pereira, Bertoni, Kandula, & Jacobs, 2010), although the degree of which it affects diabetes incidence varies with ethnicity.

In terms of anthropometrics, there was a statistically significant, albeit slight, difference in the mean BMI in the male subjects (Table 3.1). The male cases had a lower BMI than the male controls (p=0.04). This pattern was evident in all ethnic groups examined, with the difference observed in the Chinese male subjects reaching statistical significance (p=0.01). One possible explanation for this observation is that patients with T2DM are often advised to adopt a healthier lifestyle, which includes a proper diet and regular exercise, in an attempt to improve glycaemic control. This practice usually leads to an improvement in their BMI. As males more often have more lean muscle mass than females (Miller, MacDougall, Tarnopolsky, & Sale, 1993), the effects of adopting a more active lifestyle is more significant in the former rather than the latter.

When analysed according to their genotypes, it was noted that the highest mean BMI was observed in the controls carrying the homozygous variant genotypes irrespective of ethnicity. However, comparison with the homozygous variant carriers in the cases did not reveal any statistically significant difference. The mean BMI in other genotype groups in both cases and controls were not significantly different. Our findings confirm other reports that SNPs in *TCF7L2* do not affect obesity status (S. Cauchi, Choquet, et al., 2008; Pecioska et al., 2010).

There was a lower proportion of cases with overweight BMI (>23kgm^{-2}) compared to the controls. In the Chinese subjects, there was a significantly lower proportion of cases with overweight BMI compared to their controls (p=0.004). This is in contrast to a previous recent report that indicate the risk of T2DM increases with increasing BMI in the Chinese (Odegaard et al., 2009). In the other ethnic groups, the proportion was similar in cases and controls. The observation in the Chinese subjects held true even if the cut-off for overweight was raised to 25kgm-2, though losing its statistical significance (p=0.08). According to

genotypes, there were no significant difference in the overweight proportion of cases and controls, irrespective of ethnicity.

BF% is used to give a true measure of fatness, with the BMI considered together with the subjects' age and gender (Duerenberg equation). There was no significant difference between the overall BF% mean. However when analysed according to ethnicity, the Chinese cases had lower BF% compared to their controls, albeit statistically insignificant (p=0.1). The reverse is observed in the other ethnic groups. Similarly when detecting excess fat (BF% >25% in males, >33% in females), it was noted that a smaller proportion of the Chinese cases had excess body fat compared to their controls. When genotype groups were compared, there were no significant differences in BF% observed in each subject groups (cases and controls), as well as between similar genotypes of both groups.

Coupled with the observation regarding BMI above, one possibility for this paradoxical observation in the Chinese is that the lower BMI and BF% in the cases was due to lower subcutaneous, rather than visceral, adiposity. Visceral adiposity has been implicated in T2DM, partly because of the higher expression of adipokines (Samaras, Botelho, Chisholm, & Lord, 2010) and inflammatory mediators (Gabrielsson et al., 2003) compared to subcutaneous adipose tissue. Another possibility for the observed higher BMI in the controls is inadequate glycaemic control in the cases. This can lead to continuous weight loss in the cases, due to catabolism of their adipose tissue and skeletal muscle to utilise free fatty acids and amino acids, respectively, for gluconeogenesis. In the long term, body weight will eventually decrease, leading to a reduction in BMI.

4.2. SNPS IN *TCF7L2* AND T2DM IN A MALAYSIAN POPULATION

Our work is the first to present data on allelic and genotype frequencies of four SNPs in *TCF7L2* in a Malaysian population (except rs7903146). Previously as was elaborated in

chapter 1, there was only one other study looking at *TCF7L2*'s influence on T2DM prevalence in a Malaysian population, which only reported one SNP (rs7903146) (Vasudevan, et al., 2009). Taken as a whole, all examined SNPs in *TCF7L2* were shown to have a role in increasing the risk of T2DM in our study population (allelic OR between 1.62 – 1.97, p between 0.0005 – 0.004). The strongest association was observed for the T allele of rs4506565 (OR 1.97, 95% CI 1.35 – 2.88). Our findings replicate the results obtained in similar studies in other Asian (Chauhan et al., 2010; Miyake, et al., 2008; H. Qiao et al., 2012), Middle Eastern (Amoli et al., 2010; Ereqat et al., 2010; Palizban, Nikpour, Salehi, & Maracy, 2011) and Caucasian populations (Gonzalez-Sanchez, Martinez-Larrad, Zabena, Perez-Barba, & Serrano-Rios, 2008; Herder et al., 2008) that *TCF7L2* polymorphisms have a role in T2DM development. The power for these observations ranged from 82.7% for rs11196205 to 96.7% for rs4506565.

Our data also showed that in a co-dominant model (heterozygous or homozygous variant versus wildtype), the heterozygous genotypes of four of the five SNPs examined increased the risk of T2DM even after adjusting for the subjects varying age, gender and BMI (ORs between 1.72 – 1.95). This effect was not seen for the homozygous variant genotypes due to the relatively low frequency of carriers. In a dominant model (heterozygous and homozygous variant versus wildtype), the variant genotypes of all the five SNPs carries a significantly increased risk of T2DM compared to the wildtype genotype, even after similar adjustments (ORs between 1.46 – 1.95). This is reflective of the significance observed for the minor alleles above.

This finding disputed several studies reporting that the increased T2DM risk carried by the minor alleles of *TCF7L2* SNPs was modulated by BMI (S. Cauchi, Choquet, et al., 2008; S. Cauchi, Nead, et al., 2008; Lukacs et al., 2012). This is further strengthened by our data on basic characteristics of subjects according to genotypes (Table 3.2), where differences

in mean BMI and BF% between the genotypes were not statistically significant for all the SNPs examined. Therefore, in our overall study population, the increased risk observed in the variant genotype carriers was not affected by BMI.

As the Malaysian population is comprised of three major ethnic groups, the effects of the SNPs were further analysed in the Malay, Chinese and Indian ethnic groups. In the Malays, none of the SNPs conferred a significantly higher risk for T2DM. This is due to the reduction in the power of the study after segregating for ethnic groups. Although the Malay sample contained the biggest number of subjects, due to the low minor allele frequencies of all the SNPs examined in both T2DM and non-DM subjects, a significant difference in allelic and genotype frequency could not be obtained. In contrast, the smaller sample size of the Indians could produce a significant observation as the minor allele frequencies were relatively higher. This finding in the Malay ethnic group is similar to the findings of just one other study in Saudi Arabia (Alsmadi, Al-Rubeaan, Mohamed, et al., 2008). A direct comparison could not be made with this population as the minor allele frequency for the examined SNPs (rs7903146 and rs12255372) was equally high in their cases and controls. In contrast, the minor allele frequency in the cases and controls in our Malay population were equally low.

In the Chinese, several of the SNPs examined showed a lower risk for T2DM (rs7903146, rs7901695 and rs4506565). As observed in the basic characteristics of study subjects (Table 3.1), where the Malay and Indian cases and controls had similar BMI, the Chinese cases had lower BMI than their controls. Therefore, this protective effect observed in this Chinese population could be modulated by obesity status. This observed protective effect of the SNPs is in contrast with other studies in the Chinese (Lin et al., 2010; M. C. Y. Ng, et al., 2007; H. Qiao, et al., 2012). However, as the sample size for our Chinese population is

very small, this protective effect, whilst worth further exploration, should be examined with caution.

Finally in the Indians, the minor allele frequencies of the SNPs were the highest among the three ethnic groups. A significant association between the SNPs and T2DM was established in four of the five SNPs examined. This observation replicated the findings of other studies in the South (Chandak, et al., 2007; Chauhan, et al., 2010) as well as the North Indians (Gupta et al., 2010). Although significant, the small number of subjects as well as unequal sample sizes between the cases and controls led to ORs with wide 95% confidence intervals.

There was a significantly high LD between rs7903146 and rs4506565 (D' 0.977), rs11196205 and rs7901695 (D' 0.901), rs12255372 and rs4506565 (D' 0.925), and rs12255372 and rs7903146 (D' 0.926). The high LD observed between rs7903146 and rs12255372 replicates observations in other studies (Kurzawski et al., 2011; Muendlein et al., 2011; Sale et al., 2007). Strong LDs between rs4506565 and the other SNPs listed above has not been published elsewhere, whereas the strong LD between rs11196205 and rs7901695 has been shown elsewhere (S. F. Grant, et al., 2006). Strong LD indicates that both the SNPs in question are randomly linked, in which presence of one SNP can predict the presence of the other.

Haplotype analysis revealed that the strongest association was observed in the LD block containing rs7903146 and rs12255372 (Table 3.5). The TT haplotype in this block is quite common, and is significantly associated with an increased risk of T2DM (OR 1.98, p=9.97e-5). Both the alleles are variant alleles of the respective SNPs. Several haplotypes in other LD blocks constructed are also quite common (more than 20%; CCG in block 2, CACC in block 3, and ACCG in block 4). These allele combinations are comprised of wildtype alleles, but were still observed to increase the risk of T2DM, albeit slightly (OR 1.2 – 1.3). To

date, there have been no reports of these combinations and how they affect T2DM risk. Most studies reporting on haplotypes of *TCF7L2* indicate that combination of variant alleles increases risk of T2DM (Gupta, et al., 2010; Miyake, et al., 2008). Combinations containing variant alleles were associated with a significantly higher risk of T2DM. However, their frequency was quite low (around 5%).

4.3. ASSOCIATION OF SNPS IN *TCF7L2* TO HbA_{1c} LEVELS IN T2DM PATIENTS ON ANTIDIABETIC TREATMENT

The results of the present study showed that glycaemic control progressively worsens even though the number of antidiabetic agent used increases. This observation may stem from the fact that patients who are inadequately controlled on one agent usually have either more severe disease, or problems with compliance to the prescribed medication or therapeutic lifestyle changes advocated to them. Both factors tend to persist despite the increase in intensity of therapy. Severity of disease can be explained to a certain degree by the existence of genetic risk factors predisposing to the disease; in this study the genetic risk factor in question is variations in *TCF7L2*. With regards to compliance to medication and lifestyle adjustment, the subjects' BMI was taken as a surrogate marker for these as non-compliance to both usually leads to a higher BMI (Snel et al., 2011).

In general, carriers of the variant alleles have worse glycaemic control compared to their wildtype counterparts. This is true for all of the SNPs examined, irrespective of treatment regime. In patients treated with a variety of oral antidiabetic agents, further analysis revealed that patients on treatment with a metformin-sulfonylurea combination displayed significant differences in HbA_{1c} levels when compared according to their genotypes. Heterozygous carriers of the five SNPs examined had significantly higher HbA_{1c} levels compared to wildtype carriers. This was not observed in the other oral antidiabetic treatment

groups. The most obvious reason for this observation is the high number of subjects treated with these combination compared to either alone, or with other antidiabetic agent combinations. After correcting for age and BMI (to take compliance to medication and lifestyle changes into consideration), the significance of the association was maintained for four of the five SNPs (exception of rs112255372). Heterozygous carriers were also 4 times less likely to achieve HbA_{1c} targets of 6.5% in three of the five SNPs examined, which are rs11196205, rs7901695 and rs4506565.

The SNPs of *TCF7L2* have been associated, in previous studies, with poorer glycaemic response and outcome when treated with sulfonylureas. Treatment naïve patients, carrying the variant genotypes of rs7903146 or rs12255372, treated with a sulfonylurea have been shown to be less likely to achieve the target HbA_{1c} within one year of starting treatment (E. R. Pearson et al., 2007). Furthermore, increasing number of variant allele carried has been correlated with increasing likelihood of treatment failure (Kimber et al., 2007). Recently, a study replicated these findings and suggested that the poorer response to sulfonylureas could be related to an increased incidence of secondary failure with these agents (Holstein, Hahn, Korner, Stumvoll, & Kovacs, 2011).

As for metformin, there has not been much research into its pharmacogenetics. Missense mutations in organic cation transport 3 (OCT3) have been shown to reduce uptake of metformin into their sites of action, namely the skeletal muscle and liver. This has led the researchers to speculate that metformin activity could be reduced in such situations (Chen et al., 2010). Mutations in OCT1 and OCT2 have also been shown to reduce uptake of metformin into hepatocytes and to reduce its clearance via the kidney (Higgins, Bedwell, & Zamek-Gliszczynski, 2012). Besides these OCT mutations, rs391300 G>A has been reported in the Chinese to influence therapy with metformin. This study showed that presence of the variant A allele led to impaired response to metformin in treatment-naïve patients, as

measured by lower reductions in fasting plasma glucose, postprandial glucose and cholesterol (Dong et al., 2011). One study that looked at therapeutic response to metformin between different *TCF7L2* genotypes found no association between the two (E. R. Pearson, et al., 2007).

The reason for the higher HbA_{1c} in variant SNP carriers in the present study is probably linked with the mechanism of these SNPs in increasing T2DM risk. The minor alleles of these SNPs were associated with a reduction in postprandial insulin secretion by way of blunting of the incretin response (Mussig, Staiger, Machicao, Haring, & Fritsche, 2010). As treatment with sulfonylureas depends on modulation of insulin secretion, the reduced insulin secreting capacity of variant allele carriers can lead to a deleterious effect on treatment response, hence higher HbA_{1c} values. Furthermore, blunting of the incretin response can also lead to an inappropriate suppression of glucagon action, leading to increased hepatic gluconeogenesis (Knop, Vilsboll, Madsbad, Holst, & Krarup, 2007) and further worsening of glycaemic control.

Another possibility for the observed higher HbA_{1c} level is related to disease severity. Carriers of the minor alleles in *TCF7L2* SNPs have been shown to have more severe disease (Kimber, et al., 2007). Besides T2DM, rs7903146 have recently been implicated to associate with an increased incidence and severity of coronary artery disease in non-diabetic subjects (Sousa et al., 2009). Interestingly however, the same study reported that in T2DM subjects, there were no similar association observed. The same SNP was associated with an increased risk of diabetic nephropathy in patients who developed T2DM early (Buraczynska, Swatowski, Markowska-Gosik, Kuczmaszewska, & Ksiazek, 2011).

Finally, an interesting observation was made in patients treated with any other combinations, i.e. combinations utilizing newer agents such as PPARγ agonists (e.g. rosiglitazone) and dipeptidyl peptidase IV (DPP-IV) inhibitors (e.g. sitagliptin) or older

agents such as α-glucosidase inhibitors (acarbose). Variant allele carriers were observed to have lower HbA_{1c} values compared to the respective wildtypes of all of the SNPs examined. However, due to the small number of subjects in this particular group (n=29) and the wide SEM observed, this observation was not statistically significant. One possible explanation of this observation is the utilization of agents that do not depend on the capacity of the beta cells to secrete insulin (PPARγ agonists and acarbose), or agents that modulate the incretins (DPP-IV inhibitors).

CHAPTER 5

CONCLUSION

5.1. OVERVIEW

This study is the first in Malaysia looking at the impact of *TCF7L2* variations on the risk of T2DM in a heterogenous population. It was found that SNPs in *TCF7L2* predispose to T2DM in the Malaysian population which is made up of the Malay, Chinese and Indian ethnic groups. This effect persisted after age, gender and BMI were considered together in the analysis. However, in the individual ethnic groups, it was found that there was no significant association between any of the SNPs with T2DM in the Malays; certain SNPs conferred a protective effect against T2DM in the Chinese; whilst the significant association persisted in the Indians. Although distinct in appearance and, to some extent, biology, the three major ethnic groups in this study population generally share a common lifestyle. Therefore it was interesting to find that in view of this, the incidence of disease is still very much influenced by the biological system; in this case the SNPs in *TCF7L2*.

Furthermore, the present study has also showed that where treatment is concerned, genetic variations in the *TCF7L2* gene has been shown to independently affect glycaemic control in patients treated with the most common antidiabetic combination used. This is probably due to an implied increase in severity of T2DM in carriers of the variant alleles. Although statistically insignificant, the trend observed in the insulin and oral therapy combination group points towards a benefit of early institution of insulin in managing the variant allele carriers. Furthermore, combinations with other newer agents could lead to a better outcome in the variant allele carriers. However, more data are needed before this conclusion can be reached.

5.2. WEAKNESS OF CURRENT STUDY

This study suffered from the relatively small sample size. Most of the genetic epidemiological studies presently gathered data from thousands of subjects. Whilst these are genome wide association studies (GWAS), the author feels that a bigger sample would strengthen the finding of this case-control study due to the relatively smaller frequencies of the SNPs examined, especially in the Malay and Chinese subjects. The findings can then be extrapolated to the general population. The presence of multiple ethnic groups is a double-edge sword; on one hand sample heterogeneity better represents the actual population, but on the other hand the already small sample size is made smaller when stratified into individual ethnic groups.

Furthermore, the data gathered on the treatment outcome could not be collected properly. Ideally, in order to measure outcome, data on HbA_{1c} should be obtained on two separate occasions: before treatment initiation and after a certain lapse period has passed. In that sense, the cross-sectional data that we have presented could only point towards disease severity rather that the superiority of one treatment regime over another. In our study, this could not be done with the sample of patients that we had because being a tertiary healthcare centre, most patients presented to the Specialist Clinic would have already been on treatment from their primary care physicians.

5.3. FUTURE STUDIES

Therefore, the author would like to make a few recommendations to utilise the findings of the present study. Firstly, patient genotyping should be considered in the clinical management of T2DM patients. Those found to carry the variant alleles of the SNPs in *TCF7L2* should be managed more aggressively in the earlier part of their disease, due to an increased disease severity and refractoriness to treatment with the common oral antidiabetic

agents in them. Failure of oral therapy should obligate the attending physician to initiate insulin therapy early in combination with the oral medications, as the trend observed in this study suggests a more favourable outcome with this regime. The probable superior benefits of early initiation of agents that modulates the incretin axis (i.e. GLP-1 agonists or dipeptidyl peptidase-IV inhibitors) should be investigated extensively in this group of patients as the mechanism conferring risk of T2DM is due to a defective incretin response.

Finally, the author would like to propose the development of a genetic cohort study where healthy individuals in a certain population are genotyped and followed up over a certain period of time to assess their true risk of developing any disease after exposure to variant alleles of interest. A similar cohort in T2DM subjects could also be established, where the main objective is to assess treatment outcomes, disease severity and survival rate.

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APPENDICES